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*The Journal of Laboratory and Clinical Medicine*

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## *ORIGINAL ARTICLES*

# ORGAN EXTRACTS*

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### HISTORICAL REVIEW

In 1888 Brieger isolated a specific, active, toxic substance from the amputated arm of a victim of tetanus. In the year following Brieger and Frankel noted the toxalbumin in broth cultures of B. Tetani. Kitasato in 1891 injected the blood of tetanus infected laboratory animals into other animals, and Nissen did the same with blood from patients dying from a tetanus infection, whereby both were able to observe its toxic action. Brieger's pupil, Immerwahr, went a step farther than his teacher and isolated a toxalbumin from the organs of infected laboratory animals which he assumed to be identical with the product from the bouillon cultures. He likewise isolated the same substance from an amputated human leg. Stern in 1892 demonstrated the toxic activity of the blood of tetanus patients fifty-four hours before their death. Then a large number of workers began to investigate the blood stream of human beings under all sorts of pathologic conditions as well as in health. Rabbits were used as recipients and the M.L.D. of normal human blood varied among some fifteen workers between 8 and 27 c.c. per kilogram of rabbit. The blood of sheep, swine, the ox and horse were likewise injected and their toxicities determined.†

Uhlenuth[1] in 1897 reviewed these divergent observations, and after amplifying laboratory trials to include a number of new diseases and pathologic conditions, concluded that positive animal reactions to the injection of blood could not be relied upon as differentiating between a normal and a pathologic con-

*From the Hygienic Laboratory, University of Michigan.

†Quoted from Uhlenuth: Ztschr. f. Hyg., 1897, xxvi, 384-397.

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dition of the donor's blood. He made his injections subcutaneously on guinea pigs and found that all blood, in greater or less quantity, caused infiltrations at the site of the injection when in small doses, with necroses followed by death when the doses were relatively large. Normal horse serum was the least toxic and almost without effect up to a dose of 20 c.c. when an easily resorbed infiltration was evident. Obduction of dead animals disclosed nothing of any importance except in the animals dying from an injection from a scarlet fever patient, when a cloudy swelling of the kidneys was noted, accompanied by incipient fatty changes.

In 1898 Brieger and Uhlenuth made inquiry into the nature of the toxic agent that seemed to be present in all bloods, and decided that it had nothing to do with the fibrin ferment of Schmidt and Pekelharing, or with any other ferment that they knew anything about, and that it did belong to the toxalbumins, since it was readily destroyed by alcohol, and was refractory to dialysis and filtration through porcelain. They next tried the effect of placing a bit of homologous tissue under the skin, and found that similar necroses developed, usually ending in the death of the animal. A physiologic salt suspension of the tissue was found to be equally effective. Greatly to their surprise, when they extended their work to the heterologous tissues, they found that the tissues of the horse, to whose serum the animals failed to react, were very powerful in their necrotic effects. On the other hand no differences could be detected in the effects of normal human or pathologic tissues of any sort. Plunging into this work with considerable energy they soon found that a more thorough preparation of the tissues before placing them under the skin permitted them to be absorbed without appreciable effects, so they dropped the work at that point.[2]

In 1909 Schenk[3] expressed a liquid from placental tissue which had a toxic action upon intravenous injection into rabbits. He was seeking some light on eclampsia. He found that a short incubation with a small quantity of serum from varying sources sufficed to completely detoxify the expressed liquid. Inactivation of the sera did not affect their ability to render harmless the extracts. He was able to secure a short immunity to as high as three lethal doses by the usual process of repeated sublethal injections.

Kraus, Volk, and Lowenstein[4] in 1911, working in the field of tuberculins thought that animals infected with tuberculosis showed a marked susceptibility to a liquid which they had expressed from the organs of tuberculous laboratory animals. However, further researches showed that there was no specific susceptibility on the part of the infected animals, and also that liquids expressed from the organs of healthy animals served to produce the same necroses and deaths.

In Dold's[5] initial paper he reviewed much of the work that has been mentioned and confirmed that of Volk and Lowenstein in particular. He worked with physiologic salt extracts and pointed out that the toxicity of the extracts varied directly with the quantity of tissue employed or inversely to the amount of salt solution used. He found that the intraperitoneal or subcutaneous dose

was from three to four times that required by intravenous injection for the same result, and that the homologous species was relatively more susceptible to the different tissue extracts than heterologous species. He further noted that extracts lost their toxicity not only by standing at room temperature for a few days, again confirming Volk and Lowenstein, but also by heating to 60° C. for not less than thirty minutes. Such heating caused the formation of a precipitate, the decrease in toxicity being parallel with the mass of the precipitate that was thrown down. Filtration through a Berkefeld also rendered Dold's extracts nontoxic; but Aronson[6] had already noted this point in 1909 in connection with an extract of beef kidneys that he had prepared for the treatment of kidney disorders. Dold also used serum for the detoxification of extracts without acknowledgment of Schenk's earlier work which provoked a polemic concerning the priority of the work on organ extracts. Schenk[7] claimed that his "Pressäfte" was a general principle and that Dold had done nothing new when he substituted extraction for pressure or extended the work to other tissues. However, Dold did determine more closely the quantity of serum that would detoxify an extract and found [31] that so small a quantity as .5 c.c. in contact with a lethal dose of the extract for fifteen minutes completely neutralized its effects. In continuing his work Dold[8] noted that all sera were not equal in their power for detoxification, but speaking broadly, the homologous serum with the tissue was most effective. No definite law could be formulated because all sera in some proportion would minimize the toxic effect of all extracts. Filtering the sera through porcelain completely eliminated their power to remove the active agent from the extracts. Dold believed the process of detoxification to partake of the nature of a ferment inhibition, pointing in support of this view to the work of Hedin[9] who had observed that the addition of egg or seralbumin to a casein-trypsin mixture hampered digestion, and that the inhibition was more complete when the order of combination was trypsin-albumin-casein. Dold further showed that complement did not enter into the neutralization process, for by appropriate titration, complement was shown to be undiminished in a serum that had been used for detoxification as compared to an unused portion of the same serum.

Incubation with kaolin removed the toxicity of the extracts, and Izar[14] later showed that brain emulsion, talcum, animal charcoal, and rice starch would in particular amounts for each substance render the extracts harmless. When Dold attempted to immunize rabbits by successive sublethal doses the animals rapidly became cachectic and died at varying intervals. The best he could do was to obtain a very feeble resistance to one lethal dose in some animals. This is in marked contrast to Schenk's earlier claim of immunity to three lethal doses of his expressed liquid.

Inquiring into the source of the toxic substance that seemed to be ubiquitous for animal tissues, Dold[10] assumed that it had its origin in either the body cells or in the lymph. He could obtain but weakly toxic extracts from washed leucocytes or erythrocytes, and injections of relatively large quantities of either the fresh cells or the cell residues after extraction, failed to produce

an effect that was at all comparable to that of the tissue extracts. The animals died, but the preliminary symptoms were not the same and the deaths were delayed a considerable time as compared to the three to eight minute deaths of the animals receiving the tissue extracts. Dold dissected ox and rabbit eyes into their components and made extracts of the separate parts. He found the toxicity of these extracts to be parallel with their cell content. The order of toxicity in a descending scale being, uvea and retina, sclera, cornea, lens, and vitreous humor, the latter two being entirely without effect. He explains the apparent discrepancy between these findings and his assertions concerning the red and white cells by calling attention to the more strict metabolic activities of the tissue cells as compared to the functions of the blood cellular elements. Cutting a rabbit's lung into three pieces, he allowed it to remain in physiologic salt solution for two hours. After removal of the lung the solution was centrifuged, and upon injection proved to be as toxic as if the tissue had been rubbed up as was his usual custom. His conclusion as to the source of the poison is best expressed by his statement: "An extractable poison is in animal tissue in a quantity proportional to their metabolic activities and lymph content."

Animals receiving a lethal dose of organ extracts present the characteristic features of the anaphylactic syndrome. However, obduction shows clots in the heart, accompanied by abdominal hyperemia and thrombi in the lungs. Dold believed the thrombi to be the cause of death. In order to test the point he added Jacoby's hirudin to a lethal dose of the extract or else gave the animal an injection of hirudin just preceding the injection of the extract. The clots were not found in the lungs in spite of the fact that the animals died. This indicated that the supposed thrombic enzyme did not play the part assumed by Dold. A further use of a more freshly prepared extract of leeches not only saved the animals, but presumably prevented the formation of the thrombi. One can therefore form his own conclusions as to the formation and influence of the thrombi, since Dold did not attempt to further clear up the point. He found that ten per cent peptone administered in varying quantities just before the injection of the extracts saved, from a considerable series of experimental animals, an average of 75 per cent.

Bauer and Wüstoff[11] considered organ extracts to be equivalent to anaphylatoxin as prepared *in vitro*. Dold proposed the same notion and even thought that tissue from anaphylactic animals yielded a more typical anaphylatoxin.[32]

Gräfenberg and Thies[12] made a physiologic salt extract of testicles and found it to be quite toxic. They were interested in the specificity of tissue reactions as compared to species reactions to such extracts. They found that males of the same species were slightly more susceptible than any normal animals; females of the same species were highly resistant. Young animals of the same species showed no sexual differences in their reactions to the extracts. The most susceptible animals to the homologous testicular extracts were castrated males and pregnant females. Sensitized animals, which they were able to secure by careful manipulation, reacted promptly to an injection of any

extract. They could find no grounds for a species specificity, but believed their work to point to a possibility of tissue specificity to testicular extracts. Izar and Faguioli[13] made a methyl alcohol extract of bovine and dog testicles and injected into rabbits and pigs a watery emulsion of the extracted lipoids. They used a long series of dilutions and observed but slight evidences of toxicity. Heating such emulsions for one-half to one hour at a temperature of 50° C. raised their toxicity very markedly. Carrying the heating on up to a temperature of 100° C. for ten minutes still caused an increase in toxicity, but if heated longer at this higher temperature, the toxicity fell below that for the unheated extracts. A previous injection of .8 to 2.5 c.c. saturated sodium chloride containing one per cent of calcium chloride protected rabbits from death in most cases and always prolonged it. Titration of complement before and after injection of their watery emulsions showed a marked decrease following the injections, and the decrease was in general parallel to the severity of the reaction to the emulsion. They confirmed Dold's failure to secure immunity.

Izar and Ascoli[30] and later Izar[14] alone, worked with lung extracts of pigeons, sparrows, and rabbits. They noted the temperature reactions and found them to be typical for the parenteral injection of proteins. They extended the attempt to protect the recipient animals by a previous or simultaneous injection of inorganic salts, using salts of barium, strontium, magnesium, potassium, ammonium, and cadmium. Such protection as they were able to observe they ascribed to the cations involved. The various salts were not uniform in their effects and they found calcium chloride the most effective, because it lowered the toxicity more and therefore kept the temperature curve more nearly normal. They found serum from well fed animals to be most potent in detoxification; although serum from starved animals was not without considerable effect in lowering toxicities and therefore assisting in the maintenance of a more nearly normal temperature curve. Later work by Izar and Patane[15] throws interesting light on Dold's conclusions concerning the source of the poison. They made the usual extract of rabbit's lung and proved its toxicity. The tissue residue from the original extraction was then washed in several liters of physiologic salt solution in successive portions, all the washings being discarded. Then the original technic was repeated on the tissue and the extract thus made proved to be nearly as toxic as the original. Again, the lungs of a freshly bled rabbit were removed and an extract made from one immediately. The other was perfused with salt solution until it was white and bloodless, and then extracted. When both extracts were tested for their toxicity, that from the perfused lung proved to be the more toxic. They found it impossible to secure a toxic extract from the dried and powdered lung with methyl alcohol, although Fischer[16] could detect no differences in the properties of a physiologic salt extract of fresh and dried tissue. Izar and Patane incubated the physiologic salt extract of lungs at 37° C. and found that the toxic principle was quantitatively bound to the precipitate that was thrown down. Continuing Izar's previous work on the protection afforded by the inorganic salts, they were able to neutralize *in vitro* by the addition of tenth normal cal-

cium chloride. However, if to such a neutralized extract they added an exact chemical equivalent of potassium oxalate the toxicity was restored. Their earlier work had shown that potassium ions were low in protection as compared to the ions of calcium.

Fischer could not confirm Dold's observation that standing a few days at room temperature sufficed for the extracts to lose their potency, as his were effective after standing for eight weeks. He asserted that there were two substances that influenced the clotting of blood; one that hastened, and another that delayed the formation of a clot. Therefore, the obduction findings will depend on which phase of these influences was predominant when the animal died. The quantity of extract was but a minor part in the actual working out of its effects, and we are told that the exact nature of the substances that control the blood clotting is as yet unknown.

Distaso[17] worked with material secured from cadavers, also with fresh tissues of laboratory animals. He shortly abandoned the cadaverous material as he satisfied himself that their toxicity was a result of a bacterial activity after death. He found tissue extracts to be heat labile, inactivated by serum, and rendered harmless by filtering through porcelain, thereby confirming Dold's work on these points. He also reported that precipitation with alcohol destroyed the toxic agent; that treatment with fibrin or gelatine markedly lowered their effects, and that dialysis completely inactivated his extracts through the loss of salts. Before abandoning the cadaverous material as a source of tissue extracts, he worked out some of their properties, and since they were almost parallel for those of a ferment such as diastase, he concluded that the active principle of extracts was a colloidal ferment. Later he made a comparative study of extracts made from the lungs and intestines of a female rabbit and her fetal young. He found that the lungs of both yielded a toxic extract, but the intestines of the mother only did so. He argued that this showed the active principles of lung and intestinal extracts to be dissimilar, and that intestines gave a toxic extract by reason of the processes of extra-uterine life. Dale and Laidlaw[19] isolated B-iminazolylethylamine from colon tissue. Its action is very similar to Distaso's intestinal extracts, but it is heat stable, so can not be the same product. Roger[18] found that bile and pancreatic fluids, injected separately or together were highly toxic for the homologous animal. Further, Roger[20] and Falloise[21] have shown that the poisonous material contained in feces is more marked in the excreta of a breast fed infant than from an adult, and that feces of a normal adult are more powerfully toxic than from a typhoid fever patient. In all cases extracts of fecal material were heat stable.

Ichikawa[22] in 1913 studied the blood clot accelerating effects of various extracts *in vitro* and found this property to run parallel with their toxicity. He was unable to confirm Dold's assertions that the extracts were most toxic for the homologous animal and that homologous serum was most effective for detoxification. He found that sodium citrate had an effect analogous to hirudin either by previous or simultaneous injection with the extracts. He evidently considered organ extracts to be equivalent, in their effects at least, to anaphy-

latoxin, for he remarks that the temperature changes produced by injection of Friedberger's anaphylatoxin and of appropriate amounts of organ extracts are identical. Injection of large, but sublethal doses of lung extract, caused marked leucopenia; smaller doses caused an incipient leucopenia which soon reversed itself and ended in leucocytosis. Blood pressure remained unaltered by the injection of sublethal doses. No agglutination or precipitation of homologous red cells by lung extracts could be observed by Ichikawa, although Embleton[23] was able to note an appreciable rise in the hemolytic power of serum from rabbits that had received a sublethal dose of some organ extract. It will be recalled that Dold was able to detoxify his extracts by heating to 60° C. for not less than one-half hour; Ichikawa confirmed this point for rabbit lung extract, but that from the lungs of the guinea pig withstood this temperature without any diminution of toxicity. Aronson,[24] in attempting to prepare anaphylatoxin without the use of serum, heated bacterial cells in physiologic salt suspension to a temperature of 65° C. and secured a toxic filtrate by centrifuging out the germ substance. If bacteria are chemically equivalent to the extracts of tissue and one can rule out the possibility of the extraction of an endotoxin, that would be heat stable to the temperature employed, it would then be in order to use Aronson's work in checking up other results with organ extracts. Ichikawa gave support to the dual nature of the extracts as suggested by Fischer, by attempting to immunize rabbits with a heat neutralized extract. The heating eliminated the toxicity, with its accompanying blood clotting changes, but the animals all developed cachexia and died just as if they had been given sublethal doses of the unheated extract.

Dold and Kodama[25] in a later work indicate that all their extracts were acid in reaction, and that they were not able to modify their toxicity by either making alkaline or slightly more acid. A precipitate settled out upon stronger acidification which was found to carry the toxic agent with it, thereby confirming Izar and Patane in a similar observation. This precipitate could be kept dry for one month and the toxin recovered by water, ether or acetone. Addition of fresh sera to the dried precipitate, followed by removal and an immediate extraction of the substance by water showed that the sera had destroyed the toxic principle. They also added their confirmation as to the effect of alcohol on organ extracts as announced earlier by Distaso. Treatment of an aqueous extract with magnesium sulphate partially salts out the toxic substance, which could be recovered salt free by dialysis. Seeking more knowledge concerning the chemical identity and nature of organ extracts, Dold found that treatment with ether caused a separation into a watery layer on the bottom, with a precipitate above, and the ether on top of all. When very strong extracts were so treated a part of the poison was found in the precipitate, but if an extract of only moderate toxicity was handled with ether the poison was found to have been destroyed. Similar treatment with acetone always destroyed the poison, regardless of what strength of extract was used. Since these same characteristics are also common to ferments, they were held to be additional proof of the probable ferment nature of the extracts.

Catapano[26] and Bittorf[27] examined a large number of extracts of animal and human tissue for their mydriatic properties. The adrenals were the most active but all tissues with exception of thyroid, spleen, and nerve yielded an extract of greater or less activity. Bingel and Strauss[28] confirmed these workers in all their findings except for the kidney and liver. All were agreed that the extracts, as far as their mydriatic properties were concerned, were stable to heating, and the last mentioned workers claimed serum to be without effect on this characteristic. Dold[29] probably had occasion to note the same effects on the eye as these workers, because he does mention a marked inflammatory process that he had observed to come on very rapidly, which slowly subsided.

## EXPERIMENTAL

First, a brief resumé of the circumstances that provoked this study will be of some interest. In the course of a series of experiments we had occasion to attempt to determine the fate of Vaughan's poisonous protein cleavage product when administered enterally to cats. The presence of the poison in the blood stream was definitely established by heart punctures following its introduction into the stomach by a tube. By withdrawals of heart blood from a dosed cat at intervals of ten to twelve minutes, followed by immediate transfer of such blood to guinea pigs intravenously, a wave of toxicity could be noted that rose to a peak in about one hour and then gradually faded away. This observation was carefully checked by the injection of normal cat's blood. Following the matter one step farther, it seemed in order to see if any of the poison could be recovered from the tissues of such an experimental animal after it had been repeatedly dosed with the poison. For reasons which need not be given here alcoholic extraction of the tissues, followed by appropriate after-treatment of the extract was the method of procedure. Obtaining an extract that was highly toxic to guinea pigs upon intravenous injection it was interpreted as evidence of poison recovery. However, upon applying the identical technic to the organs and tissues of a normal cat an extract was secured that was equally toxic to the recipient guinea pigs, as the extract from the tissues of a dosed cat. Blocked, for the time at least, by this unexpected result, a study of the toxic properties of organs and tissues was undertaken.

*Methods Employed.*—It will save repetition if the details of the method of extracting the tissues are given at this time. What we have already referred to as the "extract," and will have frequent occasion to refer to again, was prepared by the following procedure:

Animals were killed by thorough exsanguination. Such tissues as were desired were immediately removed and placed in separate receptacles. They were washed, under the tap, free of all blood, and all fat and extraneous tissues were removed from each organ. The stomach and intestines were opened up and well cleaned. By the use of an ordinary household food chopper the tissues were ground to the consistency of a fine sausage. In this condition the tissues were placed, always in separate vessels, under absolute alcohol. The quantity of alcohol was 2 c.c. for each gram of the moist ground tissue. For this purpose Erlenmeyer flasks were used, stoppered with a plug of cotton.

They stood at room temperature with their contents for periods varying from two days to five weeks, occasionally being shaken. At the conclusion of this extraction period with the alcohol it was recovered. This was accomplished by pouring out the flasks upon a clean unstarched gauze supported in a funnel. This funnel was so arranged that the fluid passed onto a qualitative filter paper supported in another funnel below the one containing the cloth. The tissue was well squeezed out by hand compression in the gauze. The alcoholic filtrate was received in a large evaporating dish. The alcohol was driven off on a steam bath, the evaporation being carried nearly to the point of dryness. The residue left in the dish was then taken up either with physiologic salt solution or with distilled water, usually the latter. Macroscopically nothing seemed to be dissolved out of the residue by this treatment. However, color was imparted to the liquid, and this varied in intensity with the tissue concerned. This liquid was cleared by filtration through paper, and the amount of water or of salt solution added was so manipulated that the final volume of the filtrate represented two grams of the original moist tissue to each c.c. The secondary or final extraction fluid was refractory to filtration. This was especially marked in the extracts from the abdominal viscera. In order to obviate this difficulty recourse was sometimes taken to passing the liquid through a plug of glass wool in order to eliminate the larger part of the material in suspension. This filtrate was centrifuged at eight thousand R.P.M. for ten to fifteen minutes. This substitution of centrifugation for filtration produced no essential change in the characteristics of the extracts other than an increased clarity. Evidently whirling at the high speed available would throw down some particles that would pass a filter paper. This was shown to be so by centrifuging an extract that had originally been filtered. A very slight deposit was observed in the centrifuge tubes, accompanied by the change in clearness. However, no change whatever was detected in the toxicity of an extract after such treatment. This final watery solution of such materials as may have been dissolved out of the residue left upon evaporation of the alcoholic extraction liquid, is what we have designated as the extract. In all cases, except as shall be hereafter indicated, it was prepared as outlined.

*Method of Testing Toxicity.*—The guinea pig was used as the animal of choice for testing toxicity. This animal reacts promptly to injections of toxic substances, and generally with consistency. However, in tabulations of experimental data that are to follow, some instances will be noted where guinea pigs of approximate weights reacted differently to equal doses of the same extract or of other toxic substance. One must therefore consider that individual variation is still playing some part even in as highly susceptible an animal as the guinea pig. This accords with the experiences of others on the same point.[33] The place of injection was into one of the external jugulars, usually the left, the Latapie animal board being employed as a holder. The vessel mentioned is reached readily and nearly bloodlessly by a properly placed superficial incision.

Since the guinea pigs used as recipients for the extracts displayed the true anaphylactic syndrome it will be in order to briefly outline the interpretation of the phases used in tabulations to describe their responses.

*Nil* means a failure to react. Included in this group are all animals that show only an accelerated breathing rate by reason of the excitement of handling. Distinct pumping was lacking.

*Light shock* indicates some peripheral irritation, accompanied by hyperexcitability, slight dyspnea and jerks.

*Moderate shock* conveys the picture of a very rapid passage of the preliminary stages immediately followed by spasms and marked air hunger. The animal if thrown did not remain prostrate, but was soon on its feet again.

*Violent shock* and *near death* are differentiated only by the severity of the prostration. In those cases where the shock is apparently of maximum intensity, but the animal after a short period of prostration recovers promptly and fully, the first term is used; but when the prostration is long drawn out, during which time there may be some question as to whether the animal is dead or alive, the latter expression is employed.

The twitching of the nose that is so characteristic of anaphylactic death was used to mark fatal termination. Figures given under the result heading in the tables always indicate the death of the animal and give the elapsed time from the completion of the injection to the last nasal twitch.

Autopsies were invariably performed and are only commented upon when the obduction shows something at variance with the typically distended lungs, beating heart, fluid blood and hyperemia of the abdominal viscera.

*Distribution of Extractable Poison in Tissues.*—It was thought desirable in the beginning to subject a wide range of tissues to the extraction process, and note the occurrence of the toxic agent. By adhering to the same technic it would be possible to make observations as to the probable quantity of the poison present in the tissues examined. The results of this phase of the problem are conveniently summarized in Table I.

The tissues represented in Table I are sufficiently heterogenous, in both respect to species and organs, to warrant the belief that the extractable poisonous agent of tissue is present in all animal tissues to a greater or lesser extent.

*Quantity of Material Extracted from the Tissues.*—When the alcoholic extract was evaporated the mass of the residue left in the dish was slight. It formed but a very thin coating on the bottom and sides of the dish. It has already been remarked that the treatment of this residue with water did not dissolve any appreciable quantity of it. It was decided to get some exact information as to the actual quantities of material being extracted by the alcohol from the tissues. We wished further to know how much of this material was dissolved by the water, and to determine the amount of solids held in solution in the extract as injected into the guinea pigs. Accordingly, measured amounts of the alcoholic extract were evaporated to dryness on the steam bath and the weight of the residues determined. The same process was carried out on the extract as injected. From these figures it was but a simple procedure to calculate the total residue from a given amount of tissue and therefore the percentage of material that was being extracted. It is obvious the same information could be obtained concerning the extract as it was injected. It will be noted that the concentration of the final extract is expressed in terms of milligrams per cubic centimeter. Table II gives the data on the point under consideration.

The data in Table II are from the tissues of the cat. They are identical with the tissues of the cat the toxicity of which is given in Table I. It is possible to make a comparative study between the amounts of solids that are being injected and the toxicity of the extract. This will be taken up later.

TABLE I

SHOWING THE DISTRIBUTION AND RELATIVE TOXICITY OF A POISONOUS EXTRACTABLE SUBSTANCE IN TISSUE

| SOURCE | | GUINEA PIG | | C.C. | RESULT |
|---|---|---|---|---|---|
| ANIMAL | TISSUE | NO. | WT. | INJECTED* | |
| Cat | Stomach | 1 | 175 | 5 | 3′ |
| | | 2 | 175 | 3 | 2′ |
| | | 3 | 180 | 1 | Light shock |
| | Intestine | 4 | 150 | 5 | 4′ |
| | | 5 | 180 | 1 | Light shock |
| | Liver | 6 | 230 | 5 | Violent shock |
| | | 7 | 190 | 3 | Moderate shock |
| | | 8 | 160 | 1 | Nil |
| | Kidney | 9 | 235 | 5 | Violent shock |
| | | 10 | 200 | 3 | Violent shock |
| | | 11 | 215 | 1 | Light shock |
| | Spleen | 12 | 160 | 5 | 2′ |
| | | 13 | 200 | 1 | Nil |
| | Lung | 14 | 245 | 5 | Moderate shock |
| | Heart | 15 | 190 | 5 | Light shock |
| | Muscle | 16 | 215 | 4 | 1′ 30″ |
| | | 17 | 205 | 2 | Near death |
| | | 18 | 250 | 1 | Nil |
| | Brain | 19 | 190 | 5 | 2′ |
| | | 20 | 200 | 2 | Nil |
| Rabbit | Muscle | 21 | 155 | 3 | 2′ |
| | | 22 | 180 | 1 | Nil |
| | | 23 | 240 | 2 | Near death |
| | Stomach | 24 | 225 | 3 | Moderate shock |
| | | 25 | 210 | 4 | 4′ |
| | Heart | 26 | 175 | 4.5 | 3′ |
| | Spleen | 27 | 205 | 4.5 | Violent shock |
| | Brain | 28 | 190 | 4 | 2′ |
| | Lung | 29 | 210 | 4 | 2′ |
| | Kidney | 30 | 205 | 4 | 4′ |
| | | 31 | 175 | 1 | Nil |
| | Intestine | 32 | 180 | 3 | Light shock |
| | | 33 | 220 | 4.5 | Moderate shock |
| | Liver | 34 | 210 | 4.5 | 1′ 30″ |
| | | 35 | 190 | 2 | Moderate shock |
| Dog | Muscle | 36 | 200 | 2 | Violent shock |
| | | 37 | 235 | 3 | Near death |
| | | 38 | 250 | 4 | 4′ |
| | Brain | 39 | 205 | 4 | 5′ |
| | | 40 | 175 | 3 | Near death |
| Guinea Pig | Stomach | 41 | 160 | 3 | Violent shock |
| | | 42 | 165 | 4.5 | 1′ 30″ Clot in heart |
| | Intestine | 43 | 175 | 3 | Near death |
| | | 44 | 215 | 4 | Near death |
| | | 45 | 185 | 4.5 | 2′ Clot in heart |
| | Liver | 46 | 200 | 4.5 | Near death |
| | Kidney | 47 | 170 | 4.5 | 1′ 30″ |
| | | 48 | 185 | 2 | Moderate shock |
| | Spleen | 49 | 200 | 4 (5 gm.) | Nil |
| | Lung | 50 | 205 | 4.5 | 30′ |
| | Heart | 51 | 200 | 4 (7 gm.) | 1′ 30″ |
| | Muscle | 52 | 250 | 4 | 2′ |
| | | 53 | 215 | 2 | Light shock |
| | | 54 | 185 | 3 | Near death |
| | | 55 | 185 | 4 | Violent shock |
| | | 56 | 180 | 4.5 | Near death |
| | Brain | 57 | 180 | 4.5 | 2′ |
| | | 58 | 155 | 2 | Moderate shock |

TABLE I—CONT'D

SHOWING THE DISTRIBUTION AND RELATIVE TOXICITY OF A POISONOUS EXTRACTABLE SUBSTANCE IN TISSUE

| SOURCE | | GUINEA PIG | | C.C. | RESULT |
|---|---|---|---|---|---|
| ANIMAL | TISSUE | NO. | WT. | INJECTED* | |
| Sheep | Muscle | 59 | 175 | 2 | 2′ |
| | | 60 | 175 | 1.5 | Moderate shock |
| Veal | Muscle | 61 | 180 | 2 | Near death |
| | | 62 | 200 | 2.5 | 3′ 30″ |
| | Liver | 63 | 210 | 4 | 4′ |
| | | 64 | 210 | 3 | 3′ |
| | | 65 | 170 | 2 | Light shock |
| Ox | Muscle | 66 | 200 | 2 | Violent shock |
| | | 67 | 180 | 2.5 | 3′ |
| | | 68 | 170 | 2 | 4′ |
| | Liver | 69 | 160 | 2 | Violent shock |
| | | 70 | 175 | 2.5 | Near death |
| | | 71 | 250 | 3 | Violent shock |
| | | 72 | 230 | 4 | 2′ 30″ |
| Hog | Muscle | 73 | 175 | 2 | Violent shock |
| | | 74 | 165 | 2.5 | 3′ |

*Each c.c. represents 2 gm. of tissue.

TABLE II

SHOWING THE AMOUNT OF MATERIAL EXTRACTED FROM THE TISSUES OF THE CAT AND CONCENTRATION IN FINAL EXTRACT

| | ALCOHOLIC EXTRACTION | | | EXTRACT AS INJECTED | | |
|---|---|---|---|---|---|---|
| Tissue | Wt. | Wt. of residue | % of tissue | Wt. of residue | % of tissue | mg. per c.c. |
| Stomach | 69 | 0.934 gm. | 1.55 | 0.283 gm. | 0.47 | 9.44 |
| Intestine | 195 | 2.621 | 1.34 | 1.772 | 0.91 | 18.17 |
| Liver | 150 | 4.85 | 3.23 | 2.315 | 1.54 | 30.80 |
| Kidney | 62 | 0.934 | 1.51 | 0.805 | 1.30 | 25.90 |
| Spleen | 23 | 0.372 | 1.61 | 0.181 | 0.79 | 15.75 |
| Lung | 52 | 0.765 | 1.47 | 0.300 | 0.58 | 11.53 |
| Heart | 25 | 0.381 | 1.52 | 0.163 | 0.65 | 13.00 |
| Muscle | 350 | 8.275 | 2.36 | 6.406 | 1.83 | 36.60 |
| Brain | 67 | 1.410 | 2.10 | 0.732 | 1.09 | 21.85 |

*Reaction of the Extracts.*—The reaction of the extracts was usually markedly acid to litmus. Occasionally an extract of stomach or of intestinal tissue would be neutral to litmus. No ready explanation of these exceptions was apparent. The color of the extracts made it necessary to use litmus in titrating their acidity. The method of titration was to add in succession small amounts of standard alkali. Between each addition of alkali pieces of both red and blue litmus paper were applied to the solution. These were arranged on a clean glass plate, each pair being placed opposite each other and in exact order of use. The addition of alkali was continued until the reaction was clearly alkaline by both pieces of litmus. The litmus paper series passed from both red at one end to both blue at the opposite end of the series. When the strips of paper were air dry they were inspected and the neutral point was determined

by selecting from about the middle of the series that pair of papers that had the same tint. The total alkali added up to this point was therefore a measure of their original acidity. In Table III the acidity is expressed in c.c. of normal alkali required to exactly neutralize 100 c.c. of the extract. In the actual titration a more dilute alkali was employed, and the results were then calculated to the basis mentioned.

TABLE III

ACIDITY OF CAT TISSUE EXTRACT

| TISSUE | C.C. NORMAL ALKALI REQUIRED TO NEUTRALIZE 100 C.C. |
|---|---|
| Intestine A | 2.88 |
| Intestine B | 1.88 |
| Liver A | 2.71 |
| Liver B | 2.40 |
| Liver C | 1.81 |
| Lung A | 0.34 |
| Lung B | 1.77 |
| Stomach A | 0.67 |
| Stomach B | 1.60 |
| Brain A | 0.40 |
| Brain B | 2.43 |
| Kidney A | 2.50 |
| Kidney B | 2.73 |
| Kidney C | 2.00 |

The letters indicate that an extract from a different animal was being tested, although from the same tissue. Guinea pigs are susceptible to injections of acid solutions. It was thought that the acidity of the extracts might be the ruling factor in their toxicity. But no lack of toxicity was found in those extracts of stomach and intestine that were found to be neutral. It was thought best to test the matter out by neutralizing an extract and then noting its toxicity. An injection of the same extract without neutralization served as the control. Some of the extracts used were the same as those reported in the preceding table and the same letters are used to designate them. Since the dilution in no case is above three per cent it is ignored in the volume of the neutralized extract that was injected.

TABLE IV

TOXICITY OF NEUTRALIZED CAT TISSUE EXTRACTS

| TISSUE | TOXICITY | | | |
|---|---|---|---|---|
| | WHEN ACID | | WHEN NEUTRAL TO LITMUS | |
| | C.C. INJECTED | RESULT | C.C. INJECTED | RESULT |
| Intestine A | 4 | 2′ | 4 | 2′ |
| Intestine B | 4 | 2′ | 4 | 3′ |
| Liver A | 4 | 1′ | 4 | Near death |
| Liver B | 4 | Violent shock | 4 | Light shock |
| Liver C | 4 | 1′ | 4 | Moderate shock |
| Stomach B | 3 | 4′ | 4 | Light shock |
| Brain B | 4 | Violent shock | 4 | Violent shock |
| Kidney B | 4 | 12′ | 4 | Near death |
| Kidney C | 4 | 3′ | 4 | Moderate shock |
| Intestine | 4 | 2′ (clotted blood) | 4 | 2′ (clotted blood) |
| Stomach | 4 | 2′ 30″ | 4 | 5′ |

Apparently the acidity is not the cause of the toxicity. A careful consideration of the data in Table IV shows that the attempt to modify toxicity by adjustment of reaction failed both ways. That is, the toxicity was not appreciably lowered or raised from what it was originally.

*Effect of Fatigue.*—A more critical survey of the data in Table I showed that muscle tissue produced an extract that seemed to more consistently hold a high toxicity than any other one tissue. This fact caused us to consider means whereby it might be possible to radically modify the muscle tissue of an animal in its physico-chemical properties. It was proposed to extract, not only the muscle, but all other tissues of the animal and observe whether any marked change had taken place in the extract of such tissues as compared to the tissues of a normal animal. Extreme fatigue was the first to be considered. A large strong cat was selected and its urine was examined for sugar. It was found to be negative. The animal was then placed in a situation that involved a moderate but continuous muscular effort. At the end of four hours the test for sugar in the urine was strongly positive. For the next few hours the sugar remained practically constant in the urine. At the conclusion of the twenty-second hour of the exertion the urine was again negative for sugar. The presence of the sugar was used as a criterion of unusual exertion. The cat was exercised for a total of ninety hours, and then exsanguinated. The tissues were prepared and extracted as usual. Table V summarizes the injection tests of the resulting extracts.

TABLE V

TOXICITY OF EXTRACTS FROM TISSUES OF A STRONGLY FATIGUED CAT

| TISSUE | GUINEA PIG | | C.C. INJECTED | RESULT |
|---|---|---|---|---|
| | NO. | WT. | | |
| Intestine | 1 | 195 gm. | 4 | 1′ |
| | 2 | 190 | 2 | Moderate shock |
| Spleen | 3 | 160 | 4 | Light shock |
| Lung | 4 | 185 | 4 | Violent shock |
| Heart | 5 | 160 | 4 | Moderate shock |
| Stomach | 6 | 190 | 4 | 3′ |
| | 7 | 160 | 3 | 3′ |
| Muscle | 8 | 195 | 1 | Nil |
| | 9 | 250 | 2 | Light shock |
| | 10 | 235 | 3 | Moderate shock |
| | 11 | 250 | 4 | Moderate shock |
| | 12 | 215 | 6 | Violent shock |
| Liver | 13 | 185 | 3 | Light shock |
| | 14 | 205 | 4.5 | Violent shock |
| Brain | 15 | 220 | 4 | 7′ |
| Kidney | 16 | 225 | 4 | Nil |

We have not as yet attempted to set up any standard of toxicity to which an extract might be compared. Therefore anything in the nature of an exact measure of toxicity is impossible. However, a consideration of the extracts whose toxicity is shown in Table V shows that their relative toxicity is rather less than the general average. This is especially true for the extract from muscle. More will be said about the interpretation of this data later.

*Effect of Inanition.*—In continuation of the attempt to modify the properties of an extract by antemortem treatment of the animal it was decided to try inanition. Two large, well-fed cats were supplied only with water. At the end of one week one cat was removed and its tissues examined.

TABLE VI

TOXICITY OF EXTRACTS FROM TISSUES OF CAT THAT HAD BEEN WITHOUT FOOD FOR SEVEN DAYS

| TISSUE | GUINEA PIG | | C.C. INJECTED | RESULT |
|---|---|---|---|---|
| | NO. | WT. | | |
| Stomach | 1 | 190 gm. | 3 | 3′ |
| | 2 | 185 | 1 | Violent shock |
| | 3 | 180 | 2 | 5′ |
| | 4 | 250 | 1 | Moderate shock |
| Muscle | 5 | 170 | 3 | Near death |
| | 6 | 255 | 4 | 2′ |
| | 7 | 180 | 3 | Near death |
| | 8 | 185 | 3.5 | Near death |
| Spleen | 9 | 165 | 4 | Violent shock |
| Heart | 10 | 280 | 4 | Violent shock |
| Kidney | 11 | 185 | 4.5 | Violent shock |
| | 12 | 270 | 5 | Violent shock |
| Lung | 13 | 280 | 4 | 4′ |
| | 14 | 200 | 3 | 3′ |
| | 15 | 185 | 2 | 2′ |
| Brain | 16 | 180 | 4 | Violent shock |
| | 17 | 190 | 5 | 4′ (clot in heart) |
| Liver | 18 | 250 | 5 | Light shock |
| | 19 | 170 | 6 | 5′ |
| Intestine | 20 | 190 | 5 | 2′ |
| | 21 | 195 | 3 | 3′ |
| | 22 | 170 | 2 | 3′ |
| | 23 | 175 | 1 | 3′ |
| | 24 | 170 | 0.5 | Moderate shock |
| | 25 | 200 | 1 | Moderate shock |

The second cat was continued in a state of inanition for a total of twenty-four days. In the course of removing the tissues it was observed that there was still some fat left under the skin and about the viscera. Due to an error in the manipulation the final extract of these tissues was made up so that each cubic centimeter represented but one gram of tissue instead of the usual two grams. If this point be kept in mind when considering the data below it will be seen that inanition has had no apparent effect on the amount of toxic material that may be extracted from the tissues of a cat.

*Second Extraction of Tissue.*—The residual tissue from the cat which had been without food for one week was subjected to a second extraction with alcohol. In order to increase the possibility of this extract resulting in a toxic product the tissue residues were placed in a mortar and well ground with clean sand before being placed under the alcohol for the second time. When the alcoholic extract was evaporated the small quantity of the residue was especially noticeable. In order to compensate for the evident lessened amount of material that had been extracted by the alcohol, the volume of the physiologic salt solution that was added was reduced so that each c.c. of the final extract represented 4 g. of the original tissue.

TABLE VII

TOXICITY OF EXTRACTS FROM TISSUES OF CAT THAT HAD BEEN WITHOUT FOOD FOR TWENTY-FOUR DAYS

| TISSUE | GUINEA PIG | | C.C. INJECTED | RESULT |
|---|---|---|---|---|
| | NO. | WT. | | |
| Stomach | 1 | 250 gm. | 4 | Light shock |
| | 2 | 210 | 6 | 1 hr. 4′ (clot in heart) |
| | 3 | 200 | 5 | 4′ (clot in heart) |
| Heart | 4 | 220 | 5 | Violent shock |
| | 5 | 235 | 3.5 | Moderate shock |
| Eye | 6 | 210 | 4 | Nil |
| Lung | 7 | 245 | 5 | Moderate shock |
| | 8 | 285 | 7 | Moderate shock |
| Kidney | 9 | 235 | 6 | Moderate shock |
| Spleen | 10 | 225 | 6 | Violent shock |
| Brain | 11 | 220 | 5 | Violent shock |
| | 12 | 215 | 7 | 5′ |
| Intestine | 13 | 215 | 5 | 4′ |
| | 14 | 215 | 4 | 4′ |
| | 15 | 240 | 3 | 8′ |
| | 16 | 210 | 2 | Violent shock |
| Liver | 17 | 225 | 4 | Light shock |
| | 18 | 210 | 6 | Violent shock |
| | 19 | 240 | 7 | Violent shock |
| Muscle | 20 | 270 | 6 | Violent shock |
| | 21 | 305 | 8 | Violent shock |

TABLE VIII

TOXICITY OF EXTRACT PREPARED BY SECOND EXTRACTION OF TISSUE

| TISSUE | GUINEA PIG | | C.C. INJECTED | RESULT |
|---|---|---|---|---|
| | NO. | WT. | | |
| Muscle | 1 | 185 gm. | 4.5 | Violent shock |
| Lung | 2 | 195 | 4.5 | Light shock |
| Kidney | 3 | 155 | 4 | Violent shock |
| Intestine | 4 | 200 | 2.5 | Light shock |
| Stomach | 5 | 180 | 2.5 | Moderate shock |

*Extraction by Ether.*—Instead of adding 2 c.c. of alcohol for each gram of moist tissue, ether was substituted in the same proportion. The further steps in the process were not altered from the general method outlined earlier. The tissue was left in the ether for one week. Upon evaporation of the ethereal extract the minute amount of residue foretold the probable outcome of the injection tests just as it had in the previous experiment dealing with a second extraction of tissue by alcohol.

It is evident that the toxic agent that is removable from tissue by alcohol is not extracted by ether under the same conditions. This fact coupled with a consideration of the ability of dilute alcohol to remove phosphatides from tissue marks the beginning of a line of experimentation that finally led to the conclusions of this work. A more detailed discussion of the line of thought involved will be reserved for later consideration. (See Table IX.)

*Extraction of Dried Tissue.*—For this experiment it was decided to use only rabbit muscle. The extracts from muscle tissue were about as free from

TABLE IX

TOXICITY OF EXTRACT PREPARED BY SUBSTITUTION OF ETHER FOR ALCOHOL IN PRIMARY EXTRACTION

| TISSUE | GUINEA PIG | | C.C. INJECTED | RESULT |
|---|---|---|---|---|
| | NO. | WT. | | |
| Intestine | 1 | 230 gm. | 5 | Nil |
| | 2 | 210 | 9 | Nil |
| Liver | 3 | 190 | 5 | Nil |
| | 4 | 210 | 9 | Nil |
| Muscle | 5 | 245 | 9 | Nil |
| Lung | 6 | 195 | 9 | Nil |
| Heart | 7 | 195 | 7 (all) | Light shock |
| Kidney | 8 | 210 | 9 | Nil |
| Brain | 9 | 200 | 9 | Nil |

technical difficulties as any, and muscle tissue could be obtained in greater quantity more readily than others. Moreover, we were becoming more thoroughly convinced that a result arrived at by the use of any one tissue could be essentially duplicated with any other. Rabbit muscle was ground finely and dried on the steam bath. By alternate grinding in a mortar and drying it was reduced to a very fine powder of uniform consistency. This dried muscle was then subjected to treatment with alcohol for five days. The quantities of alcohol and of water used in making up the final extract were based upon the weight of the moist tissue, and were not modified from the usual quantities employed.

TABLE X

TOXICITY OF ALCOHOLIC EXTRACT OF DRIED RABBIT MUSCLE

| GUINEA PIG | | C.C. INJECTED | RESULT |
|---|---|---|---|
| NO. | WT. | | |
| 1 | 230 gm. | 3 | Nil |
| 2 | 270 | 4 | Nil |
| 3 | 280 | 6 | Nil |
| 4 | 275 | 10 | 2′ |
| 5 | 240 | 8 | 2′ |
| 6 | 275 | 7 | 2′ |
| 7 | 250 | 6 | Nil |

The striking thing about this data is the sharp break in the toxicity. It is noticed that any quantity above six cubic centimeters is fatal, whereas all doses below that amount are without effect. This is different from the graded effects that are usually shown by the injection of sublethal doses of extracts.

A portion of this extract was evaporated to dryness and the concentration of the solids determined. It was found to be 9.4 mg. per c.c. This is equivalent to but .4 of one per cent of the original tissue, and is less than any other like determination so far made. We will in this connection give another method for the concentration of solids that will soon be the subject of fuller discussion. The residue left in the crucible in which the extract was evaporated for the determination of the total solids was ignited to a white ash and its weight determined. It was found to represent .6 mg. per c.c. of the extract, or .03 of one per cent of the original tissue.

*Inorganic Material in Tissue Extracts.*—We have already given some typical figures concerning the total solids that are held in solution in the extracts of various tissues. While engaged on that point the possibility of the presence in the extracted material of a portion at least of the inorganic constituents of tissues was realized. It was but a simple matter to ignite the residue left upon evaporation of a measured portion of the extracts and note the ash left in the crucible. Strong heat was required to eliminate all carbon from the ash. It was found by several trials that ignition of the residue from 25-50 c.c. of various extracts left an appreciable ash in the crucible. In the course of some further work we will give more exact expression to the quantity of the ash.

Speculation as to the chemical constituency of such an ash was hardly necessary. In the first place it is evident that nothing could be present that had not been derived from the tissues. By reference to König's analytical work it was possible to note what the theoretical composition should be for all the tissues under examination. A part of the chlorides probably would be volatilized by the temperature of ignition, which at the same time would convert the principal element phosphorus to a pyrophosphate salt with the potassium, calcium, magnesium and other basic elements present. The ash was slowly and nearly totally soluble in water, the resulting solution being alkaline to litmus. The slight insoluble portion was at one time filtered from a solution of an ash and the volume brought back to exactly that of the original extract. A small dose of this solution was then injected into a guinea pig intravenously. A typical anaphylactic shock and death ensued. The ash in this instance had been derived from an extract of cat muscle. The amount of material in solution of the ash was found to be equivalent to 8.1 mg. per c.c. To state the same thing in another form, that is, in terms of tissue, since one c.c. of the ash solution was equivalent to 2 g. of the tissue, the inorganic ash was equivalent to 4.05 mg. per gram of tissue. Injection of 2.5 c.c. of this solution was fatal in two minutes. In the following tabulation the toxicity of the ash solutions derived from the tissues is given in comparison to the toxicity of the original extract. All the extracts were of uniform tissue value; each c.c. represented 2 g. of tissue. There was some variation in the concentration of the ash solutions derived from the extract residues. The tissue value of the ash solution of the cat muscle and of both the rabbit muscles was the same as the extract, 2 g. of tissue per c.c. The tissue value of the remaining four ash solutions was four grams per c.c. or just twice that of the others.

Consideration of the data in Table XI shows that the inorganic constituents of tissue extracts when reduced to an ash by strong ignition and then injected in water solution have a toxicity equal to in most cases and greater in some than the original extract. It is realized that the toxic agent of an ash solution can not be identical with that of the extract. There is one striking feature noted above that undoubtedly corroborates this point. It will be observed that autopsy shows that the lungs of all animals dying from an injection of ash solution are collapsed. This condition is directly opposed to the distended lungs found after death from the original extract. The contrast was very

TABLE XI

SHOWING TOXICITY OF ASH SOLUTIONS AND COMPARING THEIR TOXICITY WITH THAT OF ORIGINAL EXTRACT

| ANIMAL AND TISSUE | M.L.D. OF ORIGINAL EXTRACT | ASH SOLUTION | | | |
|---|---|---|---|---|---|
| | | GUINEA PIG | | C.C. INJECTED | RESULT |
| | | NO. | WT. | | |
| Cat muscle | 4 c.c. | 1 | 200 gm. | 2.6 | Light shock |
| | | 2 | 180 | 2.3 | 2′ lungs collapsed |
| Sheep muscle | 2 | 3 | 150 | 1 | 4′ lungs collapsed |
| | | 4 | 175 | 1 | Near death |
| | | 5 | 155 | 1 | Violent shock |
| | | 6 | 180 | 1.25 | 3′ lung collapsed |
| Veal muscle | 2.5 | 7 | 160 | 1.5 | 2′ lungs collapsed |
| | | 8 | 210 | 1.5 | 5′ lungs collapsed |
| | | 9 | 200 | 2 | 2′ lungs collapsed |
| | | 10 | 185 | 1 | Nil |
| | | 11 | 190 | 1.5 | Moderate shock |
| Beef muscle | 2 | 12 | 170 | 2 | 2′ lungs collapsed |
| | | 13 | 165 | 1 | 1′ lungs collapsed |
| Hog muscle | 2.5 | 14 | 200 | 1.5 | 5′ lungs collapsed |
| | | 15 | 180 | 1 | Nil |
| | | 16 | 200 | 1.5 | Violent shock |
| | | 17 | 180 | 1.5 | 2′ lungs collapsed |
| Rabbit muscle A | 3 | 18 | 260 | 3 | Violent shock |
| | | 19 | 230 | 4 | 2′ lungs collapsed |
| Rabbit muscle B | 3 | 20 | 260 | 3 | 2′ lungs collapsed |
| | | 21 | 215 | 2 | 2′ lungs collapsed |
| | | 22 | 225 | 1.5 | Violent shock |

marked. The distention on the one hand seemed to be nearly always maximum, while the collapse was apparently to the minimum. No other differences were observed upon autopsy of the animals killed by the ash solution.

The toxicity of the ash solutions turned our attention to the inorganic elements of the extracts. It was beyond reason to believe that the toxic agents of the extracts and of the ash solutions were the same. However, there is another possibility that could not be so easily disposed of. The exact condition of the inorganic material in living tissue is as yet uncertain. The probabilities are that most of it is in some sort of combination with the organic or nitrogenous portion of the tissues. Tests for acid radicals in the extracts were weakly indicative of their presence. The nature of the extracts made the interpretation of such tests precarious. So it must remain as doubtful whether the inorganic material of the extracts, acting as such, had any part in the original toxicity. This possibility is the subject of an experiment that will be reported later. However, it is not outside the realm of possibilities that the inorganic constituents in whatever combination they might have been, in the extracts, were the cause of their toxicity. This latter idea was therefore tested out experimentally.

*Comparison of Alcoholic and Water Extracts.*—It will be recalled that this study was evolved out of an attempt to recover Vaughan's poisonous protein cleavage product from the tissues of a cat that had been dosed enterally with the poison. Alcohol was employed as the extracting medium for two reasons. The first was the belief that the protein coagulating power of the alcohol would tend to eliminate much of the extractives of tissue. Secondly, the poison in question is more soluble in alcohol of any concentration than in

water. It was therefore expected that the differential action of the alcohol would favor its recovery from the tissue if it was present. The fact that when the absolute alcohol was added to the moist tissue it was no longer absolute was appreciated. Using the commonly accepted figures for the proportion of water in tissues and assuming an intimate intermingling of the water with the added alcohol it became about seventy-three per cent alcohol. This dilute alcohol was evidently extracting from the tissues a considerable portion of the inorganic part in whatever form it may be in the tissue. It was assumed that the inorganic material might be more soluble in water than in dilute alcohol. If this assumption should prove correct and the inorganic materials were in any manner concerned with the toxicity of the extracts, it would result in a more toxic extract. Hence it was decided to use water as the primary extracting fluid in place of the alcohol. Rabbit muscle was the tissue first subjected to such extraction. It was thought proper to make the first extraction parallel a portion of the same tissue by the alcoholic method. Then a comparison of the essential characteristics of the resulting extracts could be made. Therefore a rabbit was stripped of its muscles and they were ground as usual. The tissue was then divided into two equal parts and one was placed under alcohol, the other under water in the same proportion, 2 c.c. per gram of tissue. To the water extract formol was added to three per cent to restrain bacterial action. The final extracts are designated below as "alcohol" and "water." It is understood that the treatment of the residues left from the evaporation of the extracting liquids was identical for both and was no different from the usual method.

TABLE XII

COMPARISON OF EXTRACTS MADE FROM RABBIT MUSCLE BY ALCOHOLIC AND WATER EXTRACTIONS

| | ALCOHOL | | | | WATER | | | |
|---|---|---|---|---|---|---|---|---|
| | GUINEA PIG | | C.C. INJECTED | RESULT | GUINEA PIG | | C.C. INJECTED | RESULT |
| | NO. | WT. | | | NO. | WT. | | |
| Toxicity of extracts | 1 | 240 | 3 | 2'* | 1 | 220 | 3 | 2' |
| | 2 | 255 | 2 | Light shock | 2 | 215 | 2 | 2' |
| | | | | | 3 | 225 | 2 | 3' |
| | | | | | 4 | 225 | 1 | Light shock |
| Toxicity of ash solution* | 1 | 260 | 3 | 2' | 1 | 200 | 2 | 2'** |
| | 2 | 215 | 2 | 2' | 2 | 210 | 1.5 | 3' |
| | 3 | 225 | 1.5 | Light shock | | | | |
| Concentration of solids | Total solids 33.4 mg./c.c. or 1.67% of tissue Ash 6.32 mg./c.c. Or .32% of tissue | | | | Total solids 36.0 mg./c.c. Or 1.80% of tissue Ash 9.5 mg./c.c. Or .48% of tissue | | | |

*Volume of ash solution equivalent to extract from which derived.
**Lungs of all animals dying from ash solution were collapsed.

Certain aspects of the above data are worthy of some consideration. It is evident that the water extraction has removed more solids from the tissue than the alcohol. The increase is 7.8 per cent. This is not a marked increase. However, the ash determination tells the real difference in the extracts. The increased quantity of inorganic material in the extract made with water is ex-

TABLE XIII

SHOWING HOW TOXICITY OF EXTRACTS CAN BE CONCENTRATED BY FREEZING

| SOURCE | | M.L.D. ORIGINAL EXTRACT | NO. OF FREEZINGS | GUINEA PIG | | LAYER AND C.C. INJECTED | RESULT |
|---|---|---|---|---|---|---|---|
| ANIMAL | TISSUE | | | NO. | WT. | | |
| Cat | Muscle | 4 c.c. | 1 | 1 | 190 | 2 top | Nil |
| | | | | 2 | 220 | 4 " | Light shock |
| | | | | 3 | 210 | 2 lower | 2′ |
| | | | | 4 | 225 | 1 " | 2′ |
| | | | | 5 | 220 | 0.5 " | Light shock |
| | | | 5 | 6 | 220 | 4 top | Nil |
| | | | | 7 | 185 | 0.5 lower | Near death |
| | | | | 8 | 210 | 1 " | 1′ 30″ |
| Cat | Liver | 5 (violent shock only) | 4 | 9 | 265 | 4 top | Nil |
| | | | | 10 | 230 | 2 lower | 5′ |
| | | | | 11 | 220 | 1.5 " | Near death |
| | | | | 12 | 205 | 1 " | Near death |
| Cat | Intestine | 5 | 4 | 13 | 210 | 4.5 top | Violent shock |
| | | | | 14 | 170 | 1 lower | 1′ |
| | | | | 15 | 180 | 1 " | 4′ |
| Rabbit | Muscle | 3 | 5 | 16 | 210 | 4 top | Nil |
| | | | | 17 | 200 | 1 lower | 2′ |
| | | | | 18 | 160 | 0.5 " | 3′ |
| Rabbit | Liver | 4.5 | 5 | 19 | 205 | 4 top | Nil |
| | | | | 20 | 170 | 1 lower | Near death |
| Rabbit | Intestine | 4.5 (moderate shock only) | 5 | 21 | 165 | 2.5 top | Nil |
| | | | | 22 | 155 | 1.5 lower | Violent shock |
| Dog | Muscle | 4 | 1 | 23 | 210 | 4 top | 7′ |
| | | | | 24 | 220 | 1 lower | Violent shock |
| | | | | 25 | 160 | 2 " | 2′ (clot) |
| Guinea pig | Muscle | 4 | 3 | 26 | 185 | 4.5 top | Nil |
| | | | | 27 | 210 | 2 lower | Near death |
| | | | | 28 | 180 | 2.5 " | 2′ |
| Guinea pig | Liver | 4.5 (near death) | 3 | 29 | 190 | 4.5 top | Nil |
| | | | | 30 | 185 | 2 lower | Violent shock |
| | | | | 31 | 180 | 3 " | Near death |
| | | | | 32 | 220 | 4 " | 3′ |
| Sheep | Muscle | 2 | 3 | 33 | 175 | 4 top | 7′ |
| | | | | 34 | 185 | 0.5 lower | Violent shock |
| | | | | 35 | 185 | 1 " | Near death |
| Veal | Muscle | 2.5 | 4 | 36 | 190 | 4 top | Nil |
| | | | | 37 | 260 | 1 lower | 3′ |
| | | | | 38 | 185 | 0.75 " | Near death |
| Hog | Muscle | 2.5 | 4 | 39 | 250 | 4 top | Nil |
| | | | | 40 | 210 | 1 lower | 3′ |
| | | | | 41 | 155 | 0.5 " | Violent shock |
| Beef | Muscle | 2 | 3 | 42 | 240 | 4.5 top | 3′ |
| | | | | 43 | 240 | 2.5 " | Nil |
| | | | | 44 | 225 | 1 lower | 2′ |
| | | | | 45 | 220 | 0.5 " | Near death |
| Rabbit | Muscle | 3 | 2 | 46 | 215 | 3 top | Moderate shock |
| | | | | 47 | 245 | 1 lower | 3′ |
| | | | | 48 | 250 | 0.75 " | Moderate shock |
| | | | 5 | 49 | 185 | 6 top | Violent shock |
| | | | | 50 | 245 | 8 " | 3′ |
| | | | | 51 | 195 | 1 lower | 2′ |
| | | | | 52 | 215 | 0.5 " | 2′ |
| | | | | 53 | 200 | 0.5 " | 1′ |
| | | | | 54 | 190 | 0.25 " | Near death |
| | | | | 55 | 230 | 0.5 " | Near death |

actly 50 per cent more than that in the regular alcoholic extract. It is also observed that the water extract has a higher toxicity than the alcoholic, which is true as well of their respective ash solutions.

*Effect of Freezing.*—A mixture of salt and finely cracked ice was prepared. Portions of extracts in large test tubes were plunged into such a freezing mixture and allowed to remain until frozen solidly. The tubes were withdrawn and their contents slowly thawed by standing at room temperature. The first time that this was done it was observed that the color of the extract was slightly deeper in the bottom of the extract and correspondingly faded out in the upper portion. The intermediate area was a transition zone between the conditions at the opposite ends. A slight coagulum occupied a position just above the lower, more deeply colored portion. By means of a bulb pipette with a fine bore stem it was possible to withdraw some of the bottom part without disturbing the upper layers of the tube. Withdrawal of the top portion was of course readily accomplished. A comparison of the toxicities of the top and bottom portions showed an increased toxicity of the lower more deeply colored layer and a decreased toxic effect of the top less deeply colored part. A second freezing and thawing augmented the separation into layers that had been initiated by the first freezing and made a sharper line of demarcation between the bottom layer and the coagulum immediately above it. The bottom layer after the second freezing occupied about one-fourth to one-sixth of the total depth of the fluid in the tubes. Repeated freezing and thawing continued the layering process until the upper one-third to one-half of the fluid was nearly water white. The most of the color was concentrated in the bottom layer. The transition zone became lessened in extent necessarily. It was no longer a true transition zone excepting upwards. The break between the lower part and the coagulum area was sharp. This maximum effect could be obtained by five or six freezings and thawings. A larger number did not carry the effect any farther. In fact it could not be carried to a better separation on account of the diffusion that went on simultaneously with the thawing of the ice.

All the extracts in Table XIII were prepared by the use of alcohol as the primary extracting fluid. The last tissue in Table XIII is the extract of rabbit muscle prepared alongside the extraction of another portion of the same muscle by water. The data concerning this extract were given in Table XII. The freezing process was applied to the water extract, it being repeated four times. The separation into an upper watery layer and a lower colored layer was remarkably complete and sharp. Further, there was no coagulum anywhere in the tube. The injection tests gave the results as shown in Table XIV.

TABLE XIV

RESULT OF FREEZING AN EXTRACT OF RABBIT MUSCLE PREPARED BY USE OF WATER ONLY

| GUINEA PIG | | LAYER AND C.C. INJECTED | RESULT |
|---|---|---|---|
| NO. | WT. | | |
| 1 | 240 | 4 top | Nil |
| 2 | 270 | 6 " | Nil |
| 3 | 230 | 1 lower | 2′ |
| 4 | 225 | 0.5 " | 3′ |
| 5 | 200 | 0.25 " | 2′ |

Portions of the top and lower layers of this extract were subjected to a determination of their solids and ash by the usual methods of evaporation and ignition. The resulting values were found to be:

| | TOP LAYER | BOTTOM LAYER |
|---|---|---|
| Total solids | 3.5 mg./c.c. | 180.4 mg./c.c. |
| Ash | 0.9 mg./c.c. | 46.7 mg./c.c. |

The ash resulting from the treatment of the bottom portion was taken up in water and the insoluble part filtered out. The volume was adjusted to twice that of the extract represented, so that each c.c. was equivalent to one gram of tissue. Injection of 0.5 c.c. of this ash solution was fatal to a 200 g. guinea pig in two minutes. The lungs were collapsed. It might be of some interest to give the figures showing how the solids in solution were concentrated in the lower layer for one more extract. The extract in question is the last one in Table XIII, made from rabbit muscle.

TABLE XV

SHOWING THE EFFECT OF FREEZING ON THE DISTRIBUTION OF SOLIDS IN RABBIT MUSCLE EXTRACT

| | TOTAL SOLIDS | ASH |
|---|---|---|
| Homogeneous extract | 30.67 mg./c.c. | 5.64 mg./c.c. |
| Lower layer after two freezings | 73.84 " " | 14.4 " " |
| Lower layer after five freezings | 118.6 " " | 26.6 " " |
| Top layer after five freezings | 7.35 " " | 1.35 " " |

The figures for the solids and ash after the five freezings would undoubtedly have been much greater had not the withdrawals of a small portion of both top and bottom layers been made for injection and determination of solids and ash after the second freezing. However, the trend of the solids to move to the bottom is well shown by the figures as they stand.

*Effect of Dialysis.*—If there should be two toxic substances in the extracts it was thought they might possibly be separated by dialysis. An extract of rabbit muscle was prepared, using alcohol as the extracting liquid. The final extract was made up with distilled water. Parenthetically, we may remark that this extract demonstrates that nothing is gained by a long continuation of the primary extraction period. In this case the tissues were left under the alcohol four weeks, yet the toxicity was no greater than if left for but two days as was done in some instances. Three c.c. killed in two minutes; 2 c.c. resulting in a violent shock only.

Collodion sacs were prepared by the Novy method and mounted on cut off test tubes of appropriate size. In each case the sacs were tested for leaks before using. They were then filled with the extract to the glass line. This point furnished a convenient mark from which to note and, if desired, to measure the endosmosis. The amount of endosmosis could be accurately determined by difference between the volume of the extract placed in the dialyzer and the volume of water required to fill the sac to the liquid line at the conclusion of the

dialysis. For the first dialysis 40 c.c. of extract was used, and was continued for 24 hours at room temperature against distilled water running so that one could count the drops as they fell from the overflow. Endosmosis was 5 c.c. The increase is ignored in all volumes and figures for concentration of solids. The toxicity of the dialyzed extract was the first point to claim attention.

TABLE XVI

TOXICITY OF DIALYZED EXTRACT OF RABBIT MUSCLE

| GUINEA PIG | | C.C. INJECTED | RESULT |
|---|---|---|---|
| NO. | WT. | | |
| 1 | 195 | 3 | Nil |
| 2 | 190 | 5 | Moderate shock |
| 3 | 200 | 8 | 3′ |
| 4 | 190 | 6 | Light shock |

As the M.L.D. of the undialyzed extract was 3 c.c. it is evident that the toxicity has materially decreased. In looking for an explanation of this, the fate of the solids in solution was determined. Measured portions of the dialyzed and undialyzed extracts were subjected to the usual process of evaporation and ignition.

TABLE XVII

EFFECT OF DIALYSIS ON CONCENTRATION OF SOLIDS

| | TOTAL SOLIDS | ASH |
|---|---|---|
| Undialyzed extract | 30.67 mg./c.c. | 5.64 mg./c.c. |
| Dialyzed extract | 5.8 " " | 0.4 " " |

The results of this experiment pointed clearly to a close relationship between the toxicity of an extract and the solids held in solution. The fact that the material in solution did in large part dialyze out so readily settled its status in the extract. It was at one time thought that the solids might be in a colloidal solution, or possibly in a larger suspension or emulsion. Inability to remove any appreciable amount of material by high speed centrifugation, accompanied by an unaltered toxicity, made the notion of a suspension or emulsion no longer tenable. The only conclusion left, that of a true solution, is made much more plausible by the results of dialysis.

However, we were no nearer the truth concerning the possibility of two substances in the extract than before. It was decided to continue the investigation of this point by varying the conditions of the dialysis or the permeability of the sacs. The following protocols give the details involved:

(a) Rabbit muscle was extracted with alcohol and made up with distilled water. The toxicity of the original homogeneous extract was:

| GUINEA PIG | | C.C. INJECTED | RESULT |
|---|---|---|---|
| NO. | WT. | | |
| 1 | 225 | 3 | 1′ 30″ |
| 2 | 250 | 2 | Near death |

A very thin sac was prepared and a portion dialyzed for seven hours. The endosmosis was very marked and required a factor of .812 to be applied to the final volume in order for it to become comparable to the original extract. This correction is made in the volumes in the injection tests that are here reported for the dialyzed extract.

TOXICITY OF EXTRACT DIALYZED SEVEN HOURS

| GUINEA PIG | | C.C. INJECTED | RESULT |
|---|---|---|---|
| NO. | WT. | | |
| 1 | 220 | 2.4 | Nil |
| 2 | 245 | 3.2 | Nil |
| 3 | 225 | 4.9 | Light shock |
| 4 | 265 | 5.7 | Moderate shock |

The values for the solids and ash in the dialyzed and undialyzed extracts were found to be:

| | TOTAL SOLIDS | ASH |
|---|---|---|
| Undialyzed extract | 33.0 mg./c.c. | 6.34 mg./c.c. |
| Dialyzed extract (7 hrs.) | 9.3 " " | 1.04 " " |

Another portion of this extract was dialyzed for twenty-four hours in an ordinary sac. The endosmosis was slight and was ignored. An injection of six and eight cubic centimeters into two guinea pigs of 200 g. was devoid of toxic effect. No determination of solids was made on this latter portion.

(b) Another extract of rabbit muscle was prepared by alcoholic extraction. Three c.c. killed a guinea pig in two minutes. A dialyzing sac was made and mounted and then permitted to become air dry by standing at room temperature. The effect on the sac is to markedly lessen its permeability. The dried sac was then filled and placed in a bath at 45° C. for two hours. No endosmosis had taken place in this time. Three c.c. were withdrawn and upon injection killed the animal in two minutes. The dialysis was discontinued, as it was evident that the sac was so impermeable that no results could be expected.

(c) Another sac of moderate thickness was prepared, and when filled with the extract was placed in a bath that was adjusted to a temperature of 50°-55° C. The dialysis was continued for two hours. The endosmosis was quite marked, amounting to 20 per cent of the original volume. This is ignored in the injection tests and figures for solids and ash determinations.

TOXICITY OF EXTRACT DIALYZED TWO HOURS AT 50-55° C.

| GUINEA PIG | | C.C. INJECTED | RESULT |
|---|---|---|---|
| NO. | WT. | | |
| 1 | 205 | 3 | Nil |
| 2 | 280 | 6 | 5′ |
| 3 | 275 | 5 | Nil |
| 4 | 290 | 6 | Nil |

The determination of solids and ash gave the following results:

| | TOTAL SOLIDS | ASH |
|---|---|---|
| Undialyzed extract | 33.4 mg./c.c. | 6.32 mg./c.c. |
| Dialyzed extract | 4.95 " " | Nil |

It is to be observed that the figures for both the total solids and the ash in the undialyzed extracts reported above in "a" and "c" are quite close together. These extracts were made from the tissue of different rabbits on separate occasions. Such agreement as this is at least indicative of a possibility of the preparation of a consistent extract by following closely the same technic. As far as these dialysis experiments are concerned, it is apparently impossible to make any separation of the constituents of an extract by differential dialysis.

*Effect of Boiling Extracts.*—On three occasions a muscle extract was boiled to observe whether there was any alteration of its toxicity. The first time an extract of cat muscle was actively boiled in a large test tube over an open flame for five minutes. A fine coagulum was formed in the extract. The boiled extract was divided into two portions and one was filtered. Then injections were made of both the filtered and the unfiltered boiled extract. As a control, injection of unboiled extract was made. No differences could be detected in the effects of the three portions.

The next extract that was boiled was also from cat muscle. It had a slight opacity. Boiling one minute caused this to disappear. No coagulum was formed in this extract. Cooling under the tap did not restore the opaque appearance of the extract. No change in toxicity could be noted. Finally an extract of rabbit muscle was boiled five minutes. A coagulum similar to that produced in the extract of cat muscle made its appearance. The boiled extract was injected both filtered and unfiltered. No differences could be observed between these two portions and an unboiled portion. We believe that these tests warrant the assertion that boiling has no effect on the toxicity of a muscle extract.

*Filtration of Muscle Extract Through a Berkefeld.*—An extract of rabbit muscle was prepared with water as the primary medium of extraction. A portion was then passed through a Berkefeld "N" filter. As far as appearances were concerned the extract came through unaltered. Table XVIII shows that the toxicity remained constant also.

TABLE XVIII

FILTERING RABBIT MUSCLE EXTRACT THROUGH A BERKEFELD

| GUINEA PIG | | C.C. INJECTED | RESULT |
|---|---|---|---|
| NO. | WT. | | |
| 1 | 170 | 1 filtered | Nil |
| 2 | 170 | 1 " | Violent shock |
| 3 | 160 | 1.5 " | 3′ |
| 4 | 170 | 1.5 " | 3′ |
| 5 | 165 | 1 unfiltered | Violent shock |
| 6 | 170 | 1.5 " | 3″ |

The high toxicity of this extract may be noted. It was prepared by the use of water as the primary medium of extraction. We have already cited the experimental data showing the essential differences between an alcoholic and a watery extraction. This extract substantiates our contentions then made concerning extracts made with water.

*Phosphates and Neutrality.*—Inasmuch as we wished to do some work on the inorganic ash it was decided to prepare a reasonably large quantity of it. Therefore 966 g. of rabbit muscle was extracted with water and the final extract made up with distilled water to 483 c.c. No test of the toxicity was made, but it was all evaporated and the residue ignited. The resulting ash was taken up in the same volume of water and the insoluble portion filtered out. This

ash solution was evaporated to dryness and the residue was scraped together, carefully ground in a mortar, dried and weighed. The product thus obtained represented the water soluble portion of the inorganic ash, that had been extracted from the rabbit muscle (966 gms.) by water. It was found to weigh 4.54 g. This was equivalent to 4.7 mg. of ash from each gram of the moist tissue. The concentration, therefore, in the water solution was 9.4 mg. per c.c. Or the ash was 0.47 of one per cent of the tissue. A one per cent solution of this ash was made up. This solution was nearly of the same concentration as the original solution of the ash when the whole quantity was in 483 c.c. of water. Calculation shows that solution to have been .94 of one per cent. The one per cent solution was perfectly clear and water white. It was alkaline to litmus and phenolphthalein. One c.c. injected intravenously into a guinea pig was fatal in four minutes. Twenty-five c.c. of this one per cent solution required .25 c.c. of normal succinic acid to discharge the pink color of the one drop of phenolphthalein indicator that had been added. The toxicity of the neutralized solution was unaltered. Portions of the solution were also carefully neutralized with acetic, hydrochloric, and sulphuric acids. In each instance the toxicity remained unchanged.

The application of qualitative chemical tests to the solution of the ash showed the presence of chloride and sulphate radicals in rather minute amounts. By the use of differential tests the pyrophosphate radical was shown to be present in considerable quantity. An attempt was made to precipitate out the pyrophosphate radical by careful titration with silver nitrate. Two methods of determining the end point were employed. One, and the most successful, was to let the precipitate settle and then carefully add another drop of the reagent, noting whether a precipitate formed at the point where it was added. The other method used was that of potassium chromate as an indicator of free silver ions. A drop of the ash solution that was being precipitated by the silver nitrate was added to a drop of chromate indicator and the presence or absence of silver chromate noted. When the end point was reached the solution was allowed to stand for some time for the precipitate to settle out. Then a few c.c. of the supernatant liquid was withdrawn by a bulb pipette. Injection of this into guinea pigs showed that the toxicity had been reduced about one-half. That is, where the M. L. D. of the original ash solution was one c.c., after precipitation 2 c.c. could be injected and one-half of the pigs lived. All animals receiving injection of the precipitated ash solution showed a shock of some severity. Not a great deal of reliance can be placed on this experiment. It was shown by appropriate controls that the slightest excess of silver nitrate would produce death. In attempting to titrate to an end point in which the presence of the added ion in an unbound condition was used as the indicator it was practically certain that sufficient of these silver ions were present to upset any rigid interpretations of the injection of such a treated ash solution. However, it is significant at least that when the pyrophosphate was precipitated the toxicity showed some decrease.

The same experiment was tried with barium chloride as a precipitant, but with less success.

The phosphorous in the ash solution was determined by a standard volumetric method. It is not necessary to give the details of the procedure, since they were the same as would be employed by anyone for the same purpose and are in any standard textbook of volumetric analysis. It was determined that the ash contained phosphorus, calculated as phosphorus pentoxide to the extent of 34.83 per cent. Extending the significance of this value, it means that injection of 3.48 mg. phosphorus pentoxide, when in the form of pyrophosphate radical, is fatal to a guinea pig.

*Toxicity of Pure Phosphate Salts.*—It was decided to test the very obvious indication just brought out concerning the toxicity of pyrophosphate radical by the use of pure salts. For these tests Kahlbaum's salts were employed. However, due to the uncertainty of hydration in phosphate salts, uniform solutions were employed, and analytical results relied upon for information concerning the exact quantity of material in solution. The data will be given in form of protocols.

SODIUM ORTHO-PHOSPHATE

A one per cent solution was prepared. It was alkaline to litmus and phenolphthalein. Injection of this solution gave the following results:

| GUINEA PIG | | C.C. INJECTED | RESULT |
|---|---|---|---|
| NO. | WT. | | |
| 1 | 170 | 1 | Nil |
| 2 | 190 | 4.5 | Nil |

Twenty-five c.c. of the solution required .7 c.c. of normal succinic acid to neutralize to phenolphthalein. Injection of 4.5 c.c. of the neutralized solution was still entirely without effect. Volumetric analysis showed phosphorus calculated as phosphorus pentoxide to be 24.9 per cent. Therefore, 11.2 mg. of phosphorus pentoxide, when in the form of orthophosphate radical is without any effect upon injection into guinea pigs.

SODIUM METAPHOSPHATE

A one per cent solution was made up. It was acid to litmus and phenolphthalein. The injection tests resulted as follows:

| GUINEA PIG | | C.C. INJECTED | RESULT |
|---|---|---|---|
| NO. | WT. | | |
| 1 | 180 | 1 | 3′ |
| 2 | 170 | 0.5 | Moderate shock |
| 3 | 185 | 0.75 | 3′ |
| 4 | 165 | 0.75 | 2′ 30″ |
| 5 | 185 | 0.5 | Light shock |

Twenty-five c.c. of this solution required .33 c.c. of normal sodium hydroxide solution to neutralize to phenolphthalein. The injection of the neutralized solution gave the following results:

| GUINEA PIG | | C.C. INJECTED | RESULT |
|---|---|---|---|
| NO. | WT. | | |
| 1 | 170 | 1 | 2′ |
| 2 | 150 | 0.75 | Violent shock |
| 3 | 150 | 0.75 | Violent shock; dead in 12 hours |
| 4 | 150 | 1 | Violent shock; dead in 12 hours |
| 5 | 205 | 1 | Violent shock; slow recovery |
| 6 | 215 | 1.25 | 2′ |

Volumetric analysis showed the presence of 57.48 per cent of phosphorus as phosphorus pentoxide. Hence for unneutralized solutions of sodium metaphosphate, 4.32 mg. phosphorus as the metaphosphate radical is fatal for the guinea pig. When the solution is made neutral to phenolphthalein the amount is increased to 7.18 mg.

SODIUM PYROPHOSPHATE

In order to make more certain that the salt was all in the form of pyrophosphate it was strongly ignited before using. A one per cent solution was employed as before. The solution was alkaline to litmus and phenolphthalein. Injection of this solution gave the following results:

| GUINEA PIG | | C.C. INJECTED | RESULT |
|---|---|---|---|
| NO. | WT. | | |
| 1 | 185 | 1 | 2′ |
| 2 | 200 | 0.5 | 2′ |
| 3 | 180 | 0.25 | Light shock |
| 4 | 235 | 0.5 | Violent shock |
| 5 | 225 | 0.5 | 10′ |

The one per cent solution was diluted with distilled water volume for volume, thus making it a .5 per cent solution. Injection of the diluted solution resulted as below:

| GUINEA PIG | | C.C. INJECTED | RESULT |
|---|---|---|---|
| NO. | WT. | | |
| 1 | 205 | 1 | 3′ |
| 2 | 195 | 0.5 | Nil |
| 3 | 215 | 1 | 3′ |

Twenty-five c.c. of the one per cent solution required .5 c.c. of normal succinic acid for neutralization to phenolphthalein. Injection of the neutralized solution showed that the toxicity was the same as before. Volumetric analysis gave 52.85 as the value for phosphorus calculated as phosphorus pentoxide. The theoretic amount for a pyrophosphate salt is 52.97 per cent. Therefore, 2.64 mg. of phosphorus pentoxide in the form of a pure pyrophosphate salt is fatal for a guinea pig. Neutralization of the solution does not alter the toxicity.

The lungs of all animals dying from an injection of any of the sodium salts or the salts recovered from the extract were collapsed, and pink in color. The blood was in all cases fluid, the heart in one timed instance continuing to beat for one hour and forty-five minutes. The parallel between the toxicity of the pyrophosphate salt and the salts from the extract is the outstanding feature of this experiment. It seems sufficiently established by this test that the toxicity of the ash from the extracts is due to their content of potassium pyrophosphate.

*Toxicity of Rabbit Muscle Extract for Rabbits.*—An extract of rabbit

muscle with an M.L.D. of 1.5 c.c. for 200 g. guinea pigs was prepared by the use of water only. Some half grown and baby rabbits being available it was decided to make a few injections of this extract and note its toxicity for the homologous animal.

TABLE XIX

TOXICITY OF RABBIT MUSCLE EXTRACT FOR THE HOMOLOGOUS ANIMAL

| RABBIT | | C.C. INJECTED | RESULT |
|---|---|---|---|
| NO. | WT. | | |
| 1 | 280 | 2.1 | Nil |
| 2 | 275 | 4.2 | 1′ 30″ |
| 3 | 1085 | 8.2 | Nil |
| 4 | 1290 | 20.0 | 2′ |

In the tests in Table XIX rabbits number one and three received an injection that was fatal for guinea pig per kilogram of weight. Rabbits number two and four received twice the fatal quantity for guinea pigs per kilogram of weight.

*Experiments with Serum.*—On several occasions during the progress of the experimental part of this work an attempt was made to modify the toxicity of extracts, by the addition of rabbit serum and guinea pig serum. As far as the use of the serum of normal animals is concerned we are prepared to state that it is entirely without effect.

By repeated small injections an attempt was made to immunize rabbits against rabbit muscle extract. The injections were one-tenth the fatal quantity per kilogram of guinea pig, calculated on the weight of the rabbits. The injections were made every other day in the ear veins. On the third injection one of the rabbits gave the typical cry of an anaphylactic rabbit and was dead in two minutes. A second rabbit died on the fifth injection, notwithstanding a reduction of the dose to one-half the amount used in the beginning. The third rabbit successfully received six injections, but lost one hundred grams in weight. The animal was kept one week after the last injection and the serum secured by the usual process of bleeding in a pipette, rod defibrination, followed by immediate whirling out of the cells. This serum, possibly containing antibodies against the extract, was then added to one M.L.D. of the same extract that had been injected into the animal, and the resulting mixture tested on guinea pigs. The results of the injections are given in Table XX. Another series of mixtures of the serum and extract was prepared and then incubated at 37° C. for one hour. Following the incubation, the tubes stood at room temperature for fifteen hours and then the injections were made. The data is in Table XXI.

It is seen that the effect of the serum is nothing more than that of dilution, for the same slight evidences of weakening of toxicity can be produced by the use of physiologic salt solution. The attempt to immunize the rabbits also shows that the effect of repeated small injections apparently becomes cumulative. However one may try to explain the deaths of the rabbits, the fact remains that they were killed by a total quantity of extract that amounts to .3

TABLE XX

TOXICITY OF RABBIT MUSCLE EXTRACT WHEN MIXED WITH SERUM OF A RABBIT THAT HAS BEEN SUPPOSEDLY IMMUNIZED

| GUINEA PIG | | C.C. EXTRACT | C.C. SERUM | C.C. PHYSIOLOGICAL SALT | RESULT |
|---|---|---|---|---|---|
| NO. | WT. | | | | |
| 1 | 190 | 1.5 | 0.5 | 0 | 5′ |
| 2 | 185 | 1.5 | 0.5 | 0 | 3′ |
| 3 | 225 | 1.5 | 1.0 | 0 | Violent shock |
| 4 | 205 | 1.5 | 1.0 | 0 | 6′ |
| 5 | 215 | 1.5 | 1.5 | 0 | Violent shock |
| 6 | 210 | 1.5 | 1.5 | 0 | 10′ |
| *7 | 250 | 1.5 | 0 | 1.5 | Violent shock |
| *8 | 200 | 1.5 | 0 | 1.5 | 2′ |
| *9 | 215 | 1.5 | 0 | 0 | 3′ |

*Controls.

TABLE XXI

TOXICITY OF RABBIT MUSCLE EXTRACT WHEN INCUBATED WITH SERUM OF SUPPOSEDLY IMMUNIZED RABBIT FOR ONE HOUR; ROOM TEMPERATURE FIFTEEN HOURS

| GUINEA PIG | | C.C. EXTRACT | C.C. SERUM | C.C. PHYSIOLOGICAL SALT | RESULT |
|---|---|---|---|---|---|
| NO. | WT. | | | | |
| 1 | 250 | 1.5 | 0.5 | 0 | Violent shock |
| 2 | 200 | 1.5 | 0.5 | 0 | 3′ |
| 3 | 205 | 1.5 | 1.0 | 0 | Violent shock |
| 4 | 210 | 1.5 | 1.0 | 0 | 5′ |
| 5 | 200 | 1.5 | 1.5 | 0 | 3′ |
| 6 | 200 | 1.5 | 1.5 | 0 | Violent shock |
| 7 | 200 | 1.5 | 2.0 | 0 | Violent shock |
| 8 | 180 | 1.5 | 2.0 | 0 | Violent shock |
| *9 | 200 | 1.5 | 0 | 1.5 | 6′ |
| *10 | 245 | 1.5 | 0 | 1.5 | Violent shock |
| *11 | 250 | 1.5 | 0 | 2.0 | Near death |
| *12 | 220 | 1.5 | 0 | 2.0 | Violent shock |
| *13 | 200 | 1.5 | 0 | 0 | 3′ |
| *14 | 210 | 1.5 | 0 | 0 | 5′ |

*Controls.

in one case and .5 in the other of the quantity that would have been required to have killed them in one single injection on the assumption that rabbits are as susceptible as guinea pigs.

## DISCUSSION

The first or historical part of this paper requires but little further consideration. The reading of it has no doubt left the impression that there is yet considerable confusion in the field of organ extracts. Direct contradiction prevails on some points and agreement on certain others; but by far the larger number of questions involved are still in the balance. The one question that all have striven to answer, directly or indirectly, is the identity of the toxic agent in organ extracts.

To reflect for just a moment on the lack of agreement among the workers in this field. By a more careful reading of the details of their work one can see a plausible reason why such variant conclusions have been reached. When

one takes into consideration the diversity of workers, and hence of technic, the wide range of materials that have been examined, and the variety of animals that have been used as recipients upon which to test toxicity, it is seen at once that sharp conflicts of opinion are inevitable. Probably the variation in the technic of extraction, together with the use of different animals as recipients are the most potent factors in this matter. The latter point we believe to have especial significance. If the guinea pig may be said to be too sensitive in its response to injections, the converse is probably true of all other laboratory animals, and especially of the rabbit, which has been used so extensively in organ extract work. To illustrate, if one injects a series of half a dozen guinea pigs with graduated doses of some toxic substance, within limits, all give some definite response to the injections. The gap between no response and an easily observable effect will be relatively small in terms of dosage. This is not so for the rabbit. They receive with equanimity relatively large doses of toxic material, even bordering on the fatal quantity. Thus it is seen how two independent operators could easily arrive at opposite conclusions. One by injecting doses into rabbits that were just under the M.L.D. would get only negative results; the other by use of a quantity but slightly larger, yet over the fatal amount, makes a positive observation. Both are correct in their observations, yet the published work seems absolutely contradictory at first reading. Our personal experience with rabbits strongly corroborates this possible explanation of some of the confusion in the literature of organ extracts.

The statement made earlier concerning the circumstances under which this study was initiated may be recalled. The extraction process that was then employed is admittedly a crude procedure. However, it resulted in a toxic extract from the tissues of a normal cat. It was the substance that was in that extract that we wished to know more about, therefore the technic by which it was obtained has been quite closely followed throughout this work. It can be readily understood, that in extracting such a complex material as tissue, that any radical change in method would result in a corresponding change in the extract. The attempt to extract the tissue with ether in place of the alcohol well illustrates the point. Or, the previous drying of the tissue, followed by alcoholic extraction, which produced an extract of such negligible toxicity. The lessened toxicity of the extract from the tissues of the fatigued cat is not so readily explained. Various factors were influencing the cells of that animal during the period of fatigue, whereby they could have been markedly modified. The exertion, lack of food, worry, and the necessity of metabolism of something as a source of energy, is a combination that has many possibilities of altering an organism. The experiment on inanition alone rules out that factor, as the cat subjected to that test showed no effects of the treatment in the resulting extract of its tissues. Without going into the theoretical aspects of the matter, it may be said that authorities are agreed that two changes would ensue in the fatigued animal. One would be a change of cell contents; a transfer of energy producing material from certain cells to others more vitally concerned with the maintenance of life, such as the heart and nervous tissues. From the

viewpoint of our purposes, the change that probably took place in the cell membranes was the more potent. This assumes of course that the toxic agent of organ extracts is a constituent of cells, and must of necessity be removed through the membranes in extraction. Even if there was no change whatever in the cell contents, the decreased permeability of the membranes is very likely. This is shown by the failure of the extraction process to remove anywhere near the usual amount of toxic substance from the tissues, judging by the results of the injections.

Up to the time when water was employed as the extracting medium the extraction process had not been aimed at any particular substance. It has already been explained how our efforts of extraction became more concerned with the inorganic constituents of the tissues. The primary extraction with water resulted in an enormous increase in the bulk of the extractives removed from the tissue. However, by carrying the evaporation of the water to absolute dryness, much of this material was so changed that it was not taken up by the next treatment with the water. The data shows the actual increase of total solids to only be about eight per cent over that contained in the extract prepared by the use of alcohol as the primary extracting liquid. The increase in the inorganic matter as determined by the ignition was approximately fifty per cent. The size of dose required to kill was reduced in the case of rabbit muscle to about one-half what it had been when alcohol was used for the extraction. It would seem that the connection between the inorganic part of the extracts, in whatever form they may be, and the toxicity is close enough to warrant the belief that the toxicity is caused by this part of the material in solution. The exact form in which the inorganic material was disposed is an unsettled question. Undoubtedly some small part of it is free inorganic salts. It was found impossible to separate the inorganic from the organic substance except by ignition. Dialysis might have accomplished this result but failed to do so. This points very strongly to the probability that the inorganic elements were in combination with something else. The matter can be considered from the standpoint of the principal radical present most advantageously. As has been shown the acid radical most abundant in the ash is the phosphorus. When one considers the possible forms of combination of phosphorus in the animal body, the so-called "phosphatides," in relation to the method of extracting the tissues, it will be seen that lecithin is the most probable combination that fits the circumstances of the extraction. Further the widespread distribution of lecithin and the extractable poison would support such a view. However, lecithin is said not to dialyze. Even its solution in water is somewhat dubious. These statements are made for reasonably pure lecithin, and it is certain that we are not working with a pure specimen of anything, whatever its identity may be. All laws of solubility and behavior of substances in solution are based on data from the behavior of the pure substance. When two or more substances, even when chemically pure, are present in a solution, the result of their status is not conformable to any fixed rule for either alone. The condition when such a complex as organ extracts are placed in water would not be the most simple. This

it is believed explains the situation with respect to our tissue extracts. Disregarding entirely what the toxic agent may be, the complexity of the material is sufficient to rule out any rigid statement concerning solubility, dialysis, or other phenomena of any one substance that may be present. One fact can not be escaped. That is that in the most toxic extracts, analysis showed over one-half of the inorganic constituents of the tissue to be present, and the toxicity rose as the ash value of the extract was increased.

Early in this work we had concluded that the toxic agent of tissue extracts was some stable substance. Anything in the nature of a ferment was excluded by the method of preparation of the extract. In the first place alcohol was used to extract the tissue; ferments are not able to withstand the concentration to which they would have been subjected. Further, some rabbit livers had been saved from time to time as the animals had been used for other purposes. They were preserved by dropping into a large bottle containing absolute alcohol at the start. The most recent of these livers had been in the alcohol for about one year. They were ground and the required amount of water added to the alcohol in which they had been kept to make the volume up to the usual 2 c.c. per gram of tissue. The final extract proved to be the most toxic for liver ever prepared; 1.5 c.c. proving fatal. Secondly, the alcoholic or water extraction liquid was heated during its evaporation, which was carried to relative dryness when alcohol was used and to positive dryness in the case of water, to a higher temperature than ferments can withstand. Then, the extracts themselves were stable on boiling. The extracts showed no deterioration on standing, either at room temperature or in the ice box. Extracts thus kept for three months showed no decrease in toxicity. Whenever an extract was kept for any length of time, it was preserved from bacterial activity by a few drops of trikresol.

The work upon the toxicity of the pure phosphate salts was for the purpose of confirming a well grounded notion as to the toxic agent in the inorganic salts derived from the extract by ignition. Gamgee, Priestly, and Larmuth[36] had noted the differences in the effects of the three forms of phosphoric acid as early as 1877, and had made a special study of the influence of the pyrophosphate salt on heart and circulation. They used rabbits and frogs, and made their injections subcutaneously and intravenously. Gardner and Symes[37] repeated their work in 1910, and confirmed the same. They ascribed the effects of the phosphoric salts to their alkalinity. Starkenstein[34] made a more minute investigation of the effects of the various acid radicals, that it is possible to obtain with the phosphoric acids, due to their polyvalency, and was also led to believe that the alkalinity of the pyrophosphate salts was responsible for their marked activity. Crawford[38] after investigating the ill results of feeding cottonseed meal concluded that the pyrophosphates produced by the method of manufacture were the cause of the deleterious effects. Thus we see that there is ample evidence that the phosphate salts, and especially the pyrophosphates are very toxic substances when introduced into the body by any channel. König[39] states that phosphoric anhydride constitutes from 36 to 48 per cent of the mineral part of

various forms of flesh. The salt that we prepared and subjected to analysis contained 34.8 per cent of phosphoric anhydride. It was necessarily in the pyrophosphate form by reason of the method of its preparation, and was undoubtedly the cause of death in animals injected with a water solution of the salts. The collapsed condition of the lungs of animals dying from injections of either pure phosphate salts or of the salts from the tissue extracts, was a very constant feature. If the statement is acceptable that an anaphylactized animal dies because air can not get out of the lungs, because of the constricted bronchioles, then it would be logical to believe that these animals died because air could not get into the lungs. The dyspnea was very marked, indicating a condition of air hunger exactly analogous to that of animals injected with organ extracts or anaphylatoxin.

The method of preparing the extracts would result in a low protein content. In fact, no satisfactory test for protein was ever made on an extract. The biuret test was negative. In view of the small amount of protein, if any, that was in the extracts, the attempt to immunize rabbits was inevitably doomed to not succeed. The toxic agent of the extracts we believe to have shown to be a chemical entity, and to act as such in the animal body. However, its apparent cumulative effects on repeated injection might be interpreted as a state of sensitization.

## SUMMARY AND CONCLUSIONS

The tissues of seven species of animals were extracted with alcohol and found to yield a toxic extract. We believe that the tissue of all animals if similarly extracted would likewise produce a toxic extract.

It was found that the extract of the tissues of the cat, which may be considered representative of all other tissues, contained solids in solution varying from .47 to 1.83 per cent of the original tissue.

The extracts were acid in reaction with the occasional exception of the extract of stomach and intestine. Adjustment of the reaction to neutrality did not alter the toxicity of the extracts.

There was some evidence that toxicity of the extracts could be modified by previous treatment of the animals, especially by fatigue. Inanition was without effect.

Ether does not extract the toxic substance from tissue.

Drying and fine division of rabbit muscle followed by alcoholic extraction gave an extract of negligible toxicity. In view of statements that are to follow, we do not believe that the toxic agent was destroyed by the drying, but rather that it was rendered insoluble in absolute alcohol.

Ignition to a white ash of the solids in extracts prepared by the use of alcohol, shows an inorganic residue of approximately .4 per cent of the tissue. Subsequent solution of this ash to the volume of the extract from which it was obtained, followed by injection, shows it to be always equally toxic, and in some cases more so, than the extract from which it was derived.

It was found that water could be substituted for alcohol as the primary extracting medium, and that a more toxic extract resulted. Further investiga-

tion of this extract showed that the total solids had been increased by the use of water about 8 per cent, and the inorganic residue by 50 per cent.

The increase in toxicity of the water extracts, as measured in terms of reduction of size of dose required to kill, was approximately parallel to the increase of the inorganic material in the extracts. We, therefore, believe that the inorganic constituents of the extracts, in whatever form they may be, are responsible for the toxicity of the extracts.

That the inorganic substances were in some sort of combination is indicated by the failure of dialysis to make any separation of organic and inorganic materials. Whatever is in the extracts dialyzed freely.

By repeated freezing and thawing the toxic portion of the extracts could be concentrated to a very small volume in the lower part of the container in which the extracts were frozen. Five or six freezings gave the maximum effect. The increase in toxicity of the lower concentrated portion was roughly equivalent to the increase of solids in solution. The lack of toxicity in the uppermost portion of such a frozen extract was almost absolute.

Boiling does not affect the toxicity of muscle extracts.

The toxic agent of muscle extracts is not removed by passage through a Berkefeld N filter.

Pyrophosphate salts are highly toxic for guinea pigs upon intravenous injection. By comparative tests between pure phosphate salts and the inorganic salts derived from the extract by ignition, it was shown that the toxicity of the ash solutions was caused by their content of pyrophosphates.

The toxicity of rabbit muscle extract could not be modified by treatment with serum, either with or without incubation. This statement applies to both serum from a normal animal and serum from an animal that had been repeatedly injected with the extract, and might therefore have been immunized against the extract.

Rabbit muscle extract is toxic for rabbits, but in a higher proportion per kilogram of weight than for guinea pigs.

The attempt to immunize rabbits by successive small injections of extract shows that the effects of the extract by this manner of injection become cumulative and are exaggerated beyond what would have been produced by a single injection of the total dosage up to the time when the animal succumbs. Lack of protein material in the extracts forbids the use of the term "sensitization" as ordinarily understood. Yet the whole phenomena is typical of such a state.

The general trend of the experimental part of this work indicates that the toxic agent in tissue extracts is a stable chemical entity or entities. We believe that the evidence is sufficient to warrant the statement that the inorganic materials of the extracts, *especially the phosphorus*, in whatever form they may be are the toxic agents of tissue extracts.

ACKNOWLEDGMENTS

I wish at this time to express my thanks to Dr. V. C. Vaughan for his kind suggestions and advice, and for his friendly interest in the progress of this

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# PHYSIOLOGIC CHANGES PRODUCED BY VARIATIONS IN LUNG DISTENTION*

## A Body Plethysmograph and Its Application to the Study of Lung Distention in Relation to Circulatory Phenomena

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IN the course of investigations including a study of the circulatory changes due to distention and collapse of the lungs, of the nervous mechanism of respiration, and of the pathologic effects of overdistention it became necessary to devise apparatus by means of which accurate records of changes in lung volume could be obtained under conditions of normal or artificial respiration. It was essential that any degree of lung distention or collapse could be maintained for a considerable length of time by methods causing changes in the intrapulmonic and intrathoracic pressures approximating as nearly as possible those occurring during natural respiration. It was also desired that the apparatus should be capable of producing rapid and rhythmic changes in lung volume while a constant mean degree of distention was maintained. It was further necessary that provision be made for recording blood pressures, stimulating or freezing nerves, injecting solutions and other procedures.

The special and excellent lung plethysmographs designed either by Cloetta[1] or by Jackson[2] did not meet the requirements of our investigations which were, however, fulfilled by an adaptation of the earlier method of Bernstein[3] which consisted in placing the animal in a hermetically sealed plethysmograph, connecting the trachea with the outside air, and recording changes in lung volume from the displacement of the plethysmographic air. Certain objectionable features of this older method are, we believe, eliminated in the apparatus which we have devised and described below, while additions have been made which render it adaptable to a much wider range of experimental work. Description is facilitated by reference to two photographs (Figs. 1 and 2) and a detailed drawing (Fig. 3).

The plethysmograph** is a wooden box with a plate-glass cover. It is constructed to accommodate a medium sized dog, and leave the minimum amount of interior air space. The sides, ends, and bottom of the box are made of two layers of wood glued together with the corners lapped and fastened with screws to render it air-tight and to prevent warping. Leaks are further guarded against by a heavy inside coating of shellac and hard paraffin. The upper edges of the sides and ends of the box are surmounted by a broad wooden flange upon which the glass cover rests. In earlier attempts to secure a tightly fitting cover, the flange was in turn surmounted by a plate glass rim embedded in plaster of Paris. Leakage between the plaster of Paris and the glass rim frequently occurred, and the glass rim was abandoned for a simplified construction in which the wooden flange was carefully trued to the surface of the plate-glass cover. An air-tight union for such pressures as our animals have been subjected to, was secured by coating the

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**The plethysmograph was made by Mr. Tudor T. Hall, mechanician for Tulane University.

flange with heavy machine oil† before placing the glass cover in position.

For convenience, operative work in preparation for an experiment is performed on a special dog board which is subsequently lowered into the plethysmograph, and which is constructed so as to diminish the interior air space as much as possible. In Fig. 2, showing a top view of the apparatus, this dog board, with

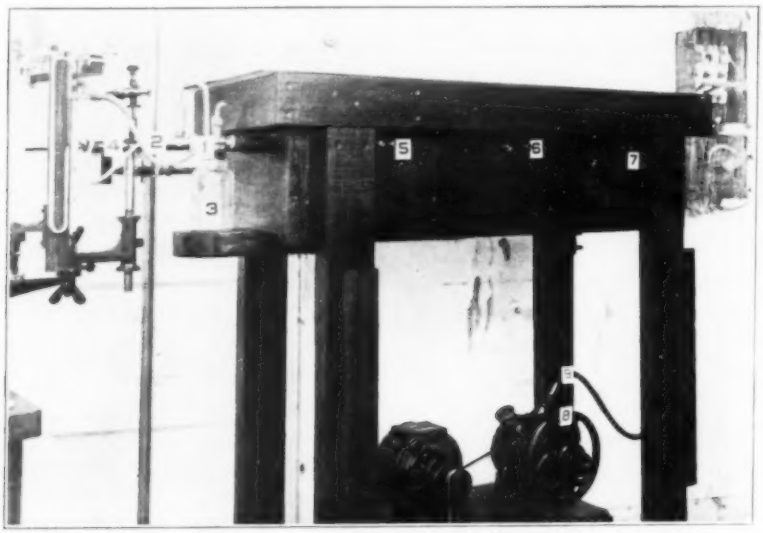


Fig. 1.—Plethysmograph showing its outside connections. *1*, Connection between mercury manometer and carotid cannula for recording blood pressure; *2*, connection with the inside of the plethysmograph for recording negative pressure changes within the plethysmograph; *3*, ether bottle connected to tracheal cannula; *4*, adjustable thumb screw valve permitting fractional pressure changes; *5* and *7*, plugs for electrical connection for stimulation of nerves; *6*, electrical connection to lamp resistance for warming interior of plethysmograph; *8*, Gaede pump; *9*, connection from exhaust pump to interior of plethysmograph.

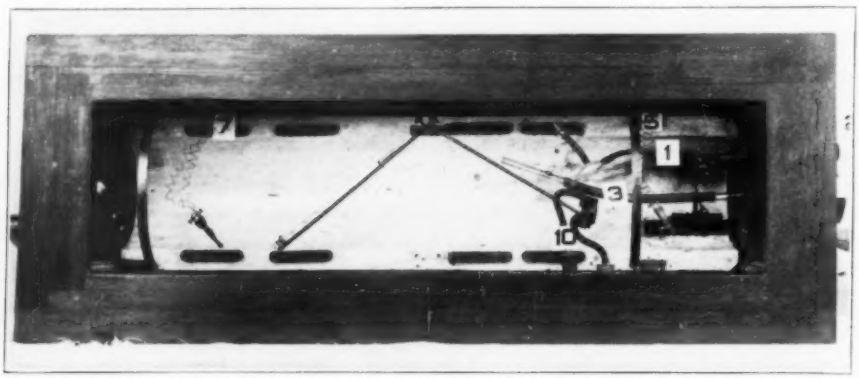


Fig. 2.—Top view of plethysmograph with plate glass cover removed, showing animal holder in position and its several connections. *1*, Blood pressure tube and carotid cannula; *2*, outlet for recording negative pressure; *3*, tracheal tube and cannula; *5* and *7*, electrical connections for stimulation of nerves; *10*, return flow tubes to conduct cold fluid for freezing nerves. Support for tubes and rods for holding thermometer, as well as the headclamp can also be seen.

its handles and rods for adjustments, can be seen in position. It is also more convenient to introduce such cannulæ as are necessary for a particular experiment before placing the animal and board in position.

†Machine oil has proved more satisfactory for our purposes than has vaseline, tallow, or lighter oils.

To make connection possible between these cannulæ and recording instruments, glass tubes pass through rubber stoppers which in turn fit openings in the wooden walls. Adjustment of the stoppers to the openings is facilitated by gas pipes driven through the openings in the wood. These pipes fit tightly and are not removable. They project exteriorly and their distal openings are more readily stoppered than openings in the wood. With the exception of the exhaust (Fig. 1, 9) all the outlets are of uniform size (No. 4 rubber stopper). This uniformity has the advantage of permitting interchange of connections. The glass tubes carried by the rubber stoppers are sufficiently long to project into the

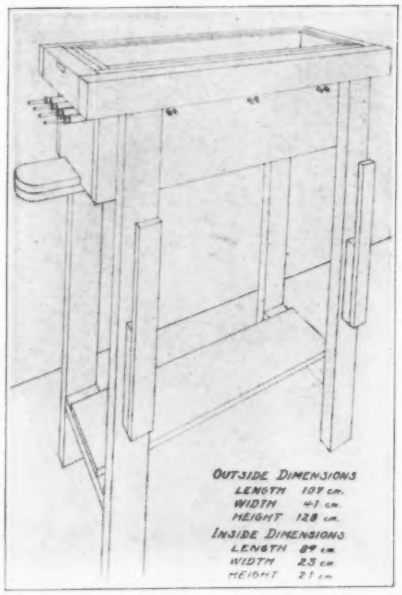


Fig. 3.—Perspective drawing of plethysmograph.

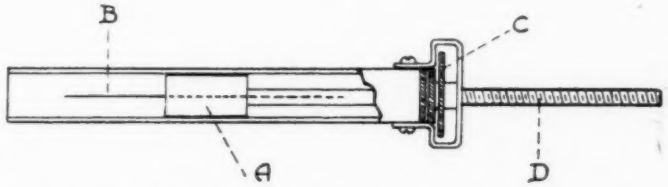


Fig. 4.—Detail drawing of the adjustable valve used to control fractional negative pressure changes within the plethysmograph. *A*, piston; *B*, slit; *C*, thumb screw; *D*, feed screw.

plethysmograph and the openings through which they pass are so placed that the tubes project interiorly above the dog board and animal.

For producing artificial distention of the lungs, the plethysmographic air is rarefied by a Gaede rotary pump. To control the plethysmographic pressure and consequent degree of lung distention, the ingress of air is varied by a regulating device (Fig. 4) of the following construction. A brass tube, 15 cm. in length and 2.25 cm. in diameter, communicates with the interior air of the plethysmograph. A slot 0.5 mm. in width is cut lengthwise through the wall of the

tube to within 2.5 cm. of its ends. This slot allows ingress of air via the cylinder to the interior of the plethysmograph. A piston accurately fitting the lumen of the cylinder prevents entrance of air from the distal end. Movement of the piston exposing a greater or shorter length of slot for the passage of air, is accomplished by a screw of small pitch passing through a threaded nut. Revolution of the nut moves the piston inward or outward. This method of controlling pressure has the advantage of obviating the influence of temperature changes within the plethysmograph, while permitting very delicate adjustment and accurate maintenance of a wide range of pressures for long periods of time.

Air is supplied to the animal's trachea through an opening in the end of the box through which, as previously described, a glass tube passes for connection with the tracheal cannula. The distal end of this tube communicates with the outside air and can be used for administration of the anesthetic. It is essential that these tubes be short in order to limit the respiratory dead space. This end is attained by an adjustable head holder on the dog board by means of which the animal's nose can be brought close to the inner wall of the plethysmograph.

In experiments necessitating artificial respiration, pulmonary ventilation is accomplished by rhythmic changes in air pressure within the plethysmograph causing alternate distention and collapse of the lungs. These pressure changes are easily effected by intermittently allowing more or less free ingress of air through one of the openings in the wall of the plethysmograph while air is being constantly withdrawn by the exhaust pump. When the opening is unobstructed it admits air freely and consequently the exhaust pump causes slight if any variation from the atmospheric pressure, but obstructions cause rarefactions of the interior air varying with the degree of occlusion. It is our belief that this method of lung inflation more closely approximates natural respiration than does intratracheal insufflation in as much as distention is accomplished, as normally, by a diminution in intrathoracic pressure.

When experiments are in progress variations of pressure within the plethysmograph are recorded by suitable apparatus communicating with the interior air. Wide variations in pressure are recorded by a mercury or a water manometer (Figs. 1 and 2) which carries a float and a recording stylus. For slight variations, a large tambour and recording lever are used. This tambour, 4 cm. in depth by 15 cm. in diameter, communicates with the interior air of the plethysmograph by means of tubing 2.5 cm. in diameter, and records the displacement of the interior air caused by natural respiration. This system is so sensitive to pressure changes and to currents of air within the plethysmograph that not infrequently heartbeats have been recorded in the respiratory tracings.

For recording blood pressure a suitable manometer, usually a mercury manometer, is connected by rubber tubing to a glass tube which fits, as previously described, an opening in the plethysmograph (Fig. 1, *1*). This system is filled with cannula fluid under pressure approximating that of an average dog and the rubber tube is clamped to prevent escape of fluid. Since this glass tube projects within the plethysmograph and would interfere with the lowering of the dog board, final adjustment is deferred until the animal is in place, when connection can be established with a cannula previously filled and inserted in the carotid ar-

tery. By similar means the pressure within the pulmonary artery may be recorded.

In the interpretation of records of blood pressure it must be borne in mind that the manometric record is affected by all factors varying the pressure within the plethysmograph and that corrections must be made by subtracting the negative pressures or adding the positive pressures. The result represents the actual blood pressure.

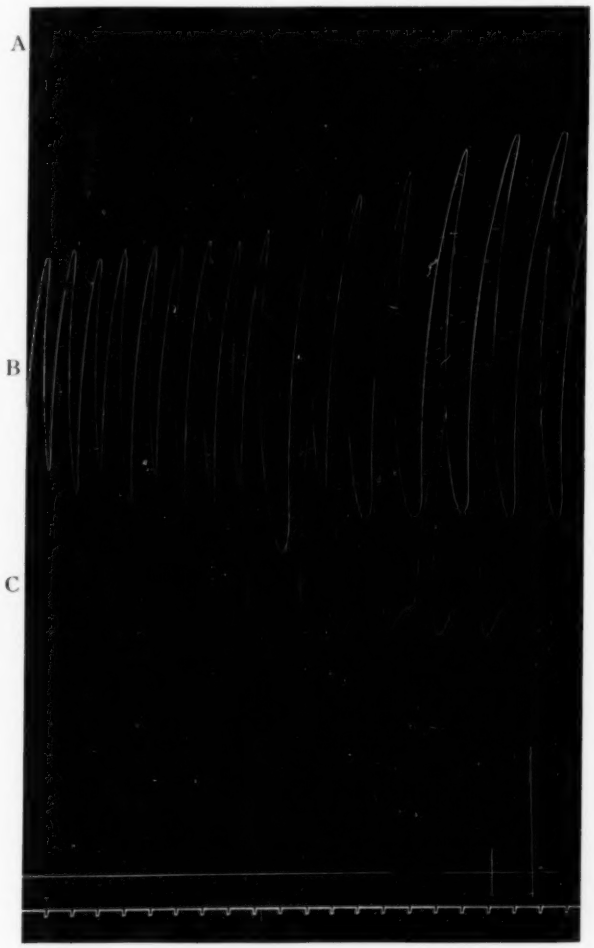


Fig. 5.—Experiment of Dec. 12, 1915. Medium-sized dog; ether anesthesia. *A*, Carotid blood pressure recorded by mercury manometer; *B*, plethysmographic curve; *C*, respiratory curve from side arm of tracheal cannula; time in seconds.

For electrical stimulation of nerves four sets of insulated binding posts on the outside of the box permit conduction of current to plugs on the interior surface. To these plugs stimulating electrodes are easily attached.

Intravenous injections are made through glass tubes fitted into rubber stoppers which in turn fit two openings in the side of the plethysmograph. These tubes are also used for establishing circulation of a freezing mixture through a suitable metallic support upon which nerves are allowed to rest.

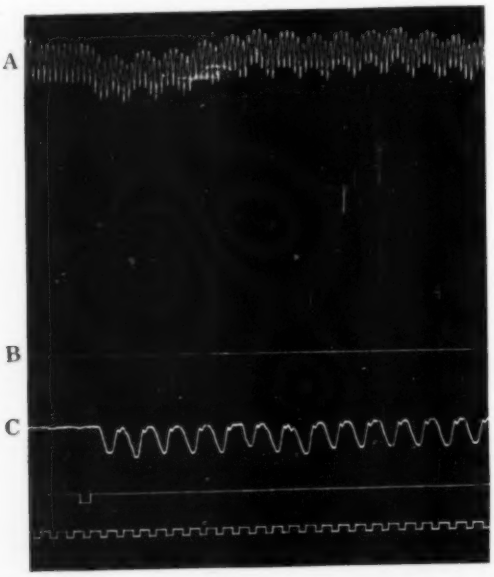


Fig. 6.—Experiment of Oct. 28, 1916. Medium-sized dog; ether anesthesia; artificial ventilation by rhythmic changes in plethysmographic pressure. *A*, Carotid blood pressure recorded by mercury manometer; *B*, abscissa for carotid blood pressure; *C*, plethysmographic pressure recorded by mercury manometer; time in seconds.

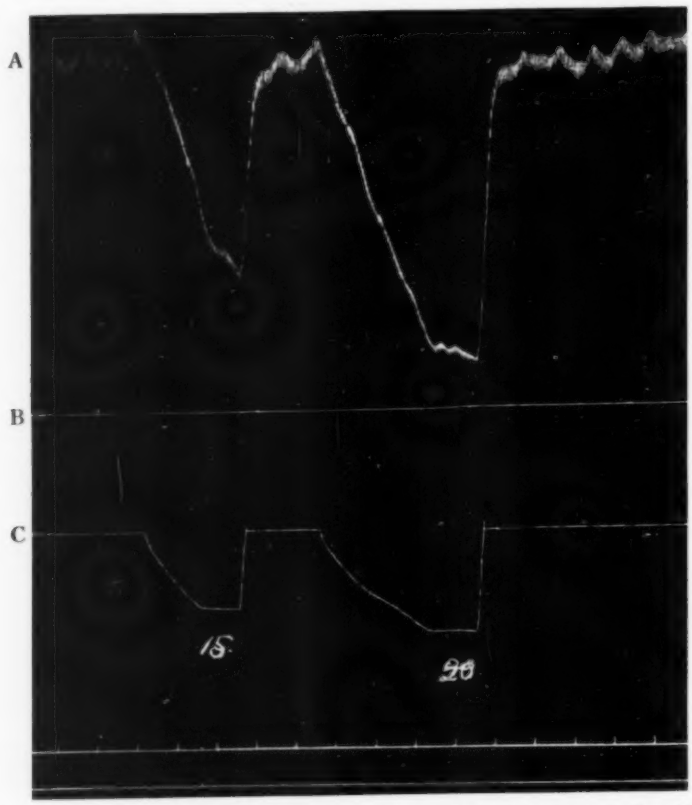


Fig. 7.—Experiment of Dec. 15, 1916. Dog 6.5 kilo; ether anesthesia. *A*, Carotid blood pressure recorded by mercury manometer; *B*, abscissa for carotid blood pressure; *C*, pressure in plethysmograph recorded by mercury manometer; time record every ten seconds.

In evidence of the adaptability and efficiency of the apparatus for recording circulatory and respiratory changes and also in maintaining constant degrees of negative pressure within the plethysmograph, five tracings are shown.

Fig. 5 shows simultaneous tracings of carotid blood pressure and two respiratory curves taken as a test of the apparatus. The period of both tambours apparently is sufficiently fast to record rapid respiration. In each of the respiratory curves the down-stroke is inspiratory and the up-stroke expiratory. The upper tracing recorded by the large tambour, is plethysmographic and the lower is from a small Marey tambour connected in the usual way with a side arm from the tracheal cannula. Both respiratory curves show changes in respiration while gradual freezing of the vagi is in progress. It will be noted that the unavoidable

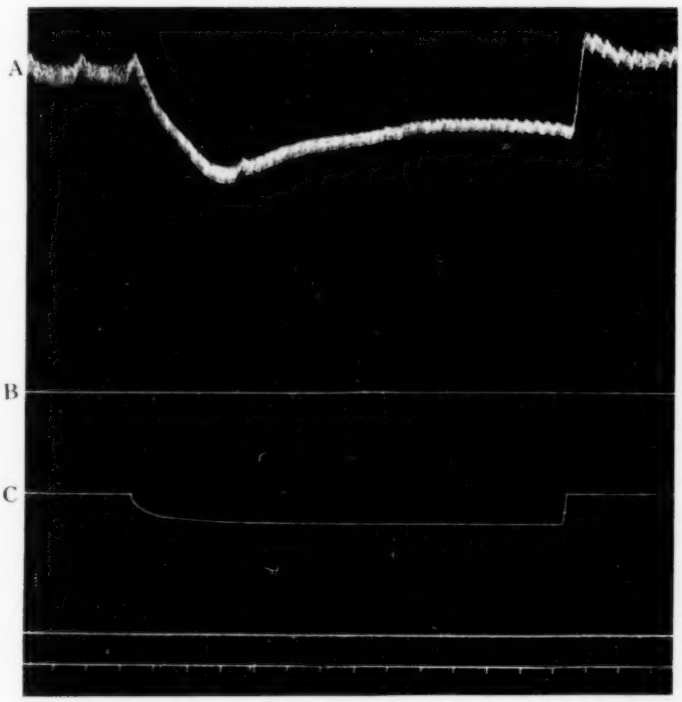


Fig. 8.—Experiment of Dec. 15, 1916. Dog 6.5 kilo; ether anesthesia. *A*, Carotid blood pressure recorded by mercury manometer; *B*, abscissa for carotid blood pressure, which is 62 mm. above the time record; *C*, pressure in plethysmograph recorded by mercury manometer; time record every ten seconds.

instrumental error in the lower tracing is absent in the upper tracing, and that the plethysmographic tracing also brings out more clearly the prolonged inspiratory phase due to abolition of function of some of the pulmonary branches of the vagi. It will further be noted that the closed system records variations in the depth of inspiration which the open system is not sufficiently accurate to register, the one recording actual volume changes and the other being affected mostly by the strength and suddenness of the expiratory blast. This closed system also has the advantage of being susceptible of calibration. The changes in blood pressure occurring as a result of freezing the vagi are not apparent in the part of the tracing shown as they occurred later.

Fig. 6 illustrates artificial pulmonary ventilation accomplished by rhythmic changes of air pressure within the plethysmograph. It will be noted that the first portion of the tracing does not exhibit in the blood pressure record the usual respiratory waves, which, however, appear with the commencement of artificial respiration which is recorded in the lower line. In this animal the chest had been previously opened.

In Fig. 7 simultaneous records of carotid and plethysmographic pressures are shown in evidence that varying degrees of negative pressure within the plethysmograph can be accurately attained and held at desired levels by the regulat-

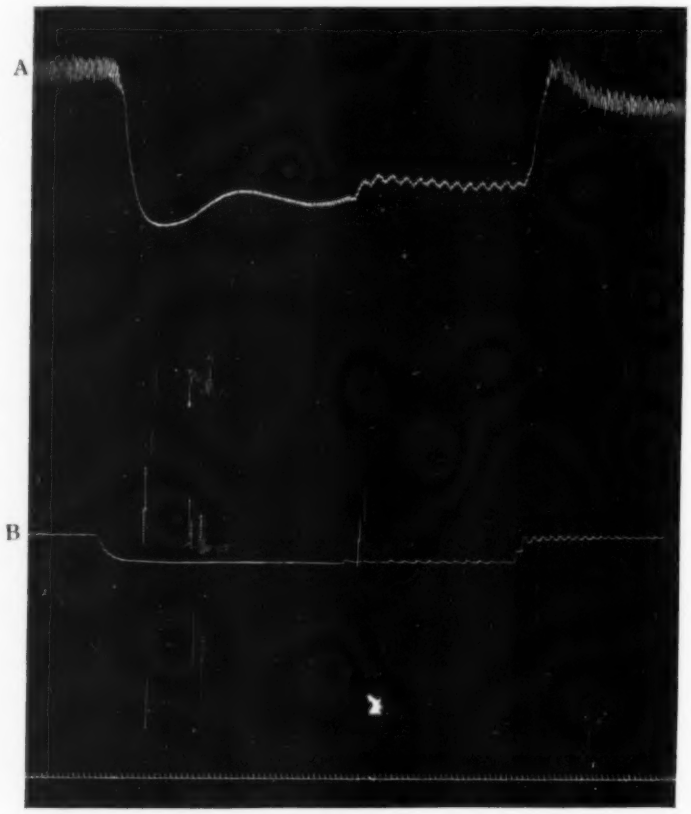


Fig. 9.—Experiment of Jan. 25, 1918. Medium-sized dog; ether anesthesia. *A*, Carotid blood pressure recorded by mercury manometer; *B*, plethysmographic pressure recorded by mercury manometer; time every second.

ing device (Fig. 4) described previously. The profound effects upon the arterial blood pressure are strikingly exemplified.

Fig. 8 shows a rapid fall in the plethysmographic pressure from zero to minus 10 millimeters of mercury and from this point during a period of 125 seconds, a further gradual fall to a minimum of minus 14 millimeters attained immediately before the release of the plethysmographic pressure. After the initial fall, the carotid pressure, in spite of this continuously falling plethysmographic pressure, shows a progressive rise of 20 millimeters of mercury within a period of 70 seconds. This higher level is maintained until the release of ple-

thysmographic pressure to the atmospheric level, when the blood pressure mounts rapidly to a level 16 millimeters of mercury above that maintained prior to the experiment. The rise in carotid pressure during the period of gradually falling plethysmographic pressure has been interpreted by us as due to systemic vasoconstriction. It was to emphasize this point that the plethysmographic pressure was gradually decreased in this experiment.

By the method of exhaustion with the Gaede pump and regulation by the device we have described, it is possible to maintain a constant degree of negative pressure in the plethysmograph for any period desired. An illustrative record of one of the shorter periods is presented in Fig. 9. In this experiment a negative pressure of $5\frac{1}{2}$ millimeters was maintained for a period of 80 seconds, but the results obtained are typical of much longer periods. There is an abrupt fall of carotid pressure, then partial recovery and subsequent slight fall—constant phenomena which occur in varying degree, within moderate ranges of negative pressure. A long period of apnea occurs as a result of lung distention. In the latter half of the tracings, however, respiratory efforts show, and also an interesting rise in blood pressure coincident with the commencement of respiration.

The fall in blood pressure noted in the tracings illustrates the profound circulatory disturbance due to lung distention. With the apparatus here described these disturbances have been the subject of investigation, the results of which have furnished material which will appear in a second communication.

We are indebted to Professor W. E. Garrey for helpful suggestions and criticism.

## SUMMARY

A plethysmograph is described which is large enough to hold a medium sized dog. It permits of varying the distention of the lungs by exhausting the interior air and the degree of fall in pressure is controlled by a device described in the text. Pulmonary and carotid pressures and other circulatory conditions as well as the pressure within the plethysmograph may be recorded manometrically. The vagi and other nerves may be frozen or stimulated, the anesthetic varied, and artificial respiration resorted to without interrupting the experiment or opening the plethysmograph. Experiments showing some of the applications and the methods and their results are briefly described.

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# *LABORATORY METHODS*

## SIMPLIFIED GAS ANALYSIS

### NO. III. THE DISSOCIATION CURVE FOR OXYGEN AND THE $CO_2$-COMBINING POWER OF BLOOD

By J. J. R. Macleod, M.B., and R. S. Lang, B.A., Toronto, Canada

FOR accurate determination of the relative amounts of reduced and oxyhemoglobin in blood exposed to atmospheres containing varying partial pressures of oxygen no method surpasses that of Barcroft and his coworkers.[1]

The principle of this method is to expose a small quantity of blood in a thin film on the walls of a relatively large cylindrical vessel (tonometer) containing a mixture of nitrogen and oxygen gases until equilibrium has become established between the partial pressure of the oxygen in the atmosphere and the absorption of oxygen by the blood. Some of the blood is then transferred to a bottle connected with a differential manometer and shaken with dilute ammonia water, by which it becomes laked and takes up its maximal load of oxygen, thereby causing shrinkage in the volume of air, the degree of which is indicated by the manometer. The oxygen-saturated blood is then shaken with ferricyanide of potassium which dislodges the oxygen and causes the pressure in the bottle to rise. From the displacement of the fluid in the manometer in the two observations, the percentage of saturation of the blood with oxygen is readily calculated.

For use by a class of students the method is not practical because of the difficulty of providing suitable mixtures of oxygen and nitrogen with which to fill the tonometer and because of the expense of the differential manometer. The first of these difficulties is overcome by exposing the blood to a partial vacuum instead of a mixture of gases, a principle which has also been applied by W. G. Macallum, who uses a modified Van Slyke pipette both as tonometer and analysis apparatus.[2] The advantage of a partial vacuum over a mixture of oxygen and nitrogen is that the partial pressure of oxygen is readily calculated from the degree to which the tonometer is evacuated as measured by a barometer. In the present method the blood after exposure to the partial vacuum is transferred to a simple form of differential blood gas manometer in which there are no glass stopcocks, pressure adjustment being made by the use of the pressure adjuster described elsewhere.[3] Even with these simplifications, the technic is by no means easy, but the great importance of having the student clearly understand the principles of the method used for securing the data necessary to plot the dissociation curve amply repays the time he devotes to the experiment. Indeed without actually doing this experiment, it is the opinion of the writers that the average student rarely acquires any clear conception of the respiratory function of the blood. After he has conscientiously performed the experiment, on the other hand, the whole problem becomes clearer, even although results that are

absolutely correct may not have been secured. A laboratory session of at least three continuous hours is essential, and the students can most profitably work in groups of three each.

## DESCRIPTION OF THE APPARATUS

*The Tonometer.*—This consists of a wide glass tube (the tonometer *T*, Fig. 1) of fairly stout glass, tapering down to narrow tubes at both ends. The capacity should be at least 200 c.c.* The narrow tubes are connected with thick-walled (pressure) rubber tubing which should be wired on to the glass tubes. The rubber tubes are closed by screw clips (1 and 2). File marks are made at

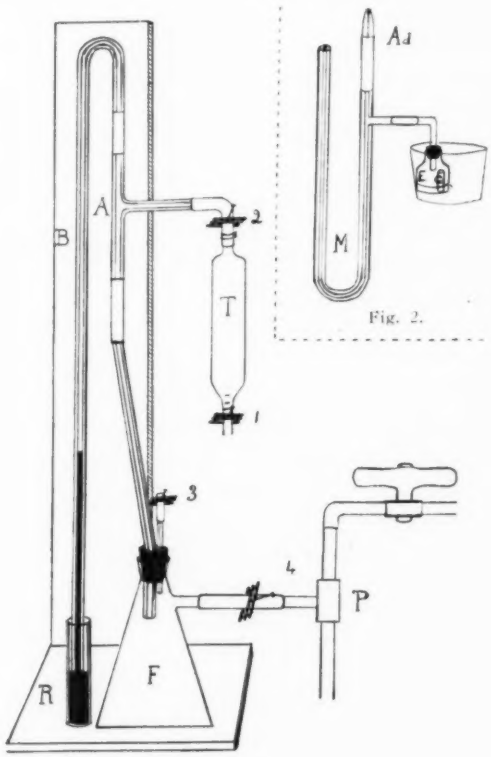


Fig. 2.

Fig. 1.

one of the tapering ends of the tonometer, the distances between them corresponding approximately to one cubic centimeter.

*The Barometer.*—This consists of a vertical, thick-walled glass tube about 1.25 meters long and of about 3 mm. bore bent on itself near one end, and with the other end dipping into mercury contained in a wider flat-bottomed (specimen) tube (the mercury reservoir) closed by a perforated cork. The barometer tube and reservoir are firmly mounted on a stand furnished with a millimeter scale which is attached to the stand in such a way that it can be adjusted to bring its zero to the surface of mercury in the reservoir, as this varies at different pressures. The free end of the barometer tube is connected by rubber pres-

*It would be preferable to use a tonometer two times as large since this would diminish errors due to the addition of the gas given off from the blood.

sure tubing to a glass T-piece (*A*), one limb of which is similarly connected to a stout-walled (filtration) flask (*F*) joined to a good water pump (*P*). A capillary tube closed by a piece of rubber tubing and a screw clip (*3*) also passes through the stopper of the flask.

*The Differential Manometer and Gas Bottle.*—This apparatus requires a piece of narrow bored glass tubing (bore 1 mm.) bent into a U-shape with one limb about 200 mm. long and the other about 150 mm. (*M* in illustration). To the shorter limb is connected a T-piece (of narrow bore tubing) which should be fused to it, although rubber pressure tubing is quite satisfactory. One limb of the T-piece is connected with red rubber tubing of moderately thick wall, closed by a pressure adjuster (Ad) as described elsewhere.* By pinching the rubber tubing lying over the lateral hole of the adjuster, the manometer is brought into communication with the outside so that the air in the bottle and manometer are brought to atmospheric pressure. The other tube of the T-piece is connected by pressure rubber tubing with a glass tube which passes through a rubber stopper that accurately fits a small wide-mouthed bottle *E* of about 15 c.c. capacity. Finally a small flat-bottomed test tube 15 mm. high and 6-7 mm. diameter is required, *I*. Instead of attaching the T-piece to the short limb of the manometer, it may be inserted in the rubber stopper, one limb being connected with the manometer—which in this case simply consists of a U-tube—and the other with the pressure adjuster. The fluid used in the manometer is clove oil.

## TECHNIC OF ANALYSIS

The tonometer is rinsed out with physiologic saline and is connected with the side tube of the barometer T-piece *A*. The pump *P* is turned on with screw clips *1* and *3* closed, but screw clip *2* open and the pressure lowered until the mercury stands at a constant level in the barometer. Screw clip *4* is closed and the mercury observed to see whether there is any leak. Provided there is none, clip *3* is cautiously opened and the mercury allowed to fall almost to the level in the reservoir (*R*); clip *2* is tightened, the tonometer, *T*, removed, and the pump turned off. Defibrinated or oxalate blood (whipped ox blood is most suitable for large classes, but in any case blood from an etherized animal must not be used) is now sucked into the tonometer, by placing some of the blood in a small evaporating dish and cautiously loosening clip *1* with the rubber tube dipping into the blood; *3* to *4* c.c. of blood should be allowed to enter the tonometer. This is then reattached to the T-piece *A* of the barometer and with clips *2* and *4* open (but *1* and *3* closed) the pump is turned on and the mercury allowed to rise as far as it will go, when clip *4* is closed and the pump turned off. Clip *3* is now cautiously opened until there is a partial pressure of about 20 mm. Hg oxygen in the tonometer.†

*This Journal, iv, 69.

†This is computed as follows: After suitable adjustment the standard barometer in the room is read and from the reading is subtracted the tension of aqueous vapor at the temperature of the room (for table see page 420 of this Journal, Vol. III). The difference gives the pressure in mm. Hg of an atmosphere of dry air. Since air contains 20.96 per cent oxygen, the partial pressure of this gas in the tonometer must be equal to $\frac{20.96}{100}$ of the difference between the height to which the mercury is raised in *B* and the corrected barometer reading. Thus, suppose the room barometer is 753.4 mm. and aq. tension 17.4 mm. the corrected barometer reading is 753.4-17—4 = 736 mm. Then $\frac{20.96}{100} \times 736 = 154.2$ mm. $O_2$.

$$20 \text{ mm. } O_2 = \frac{20 \times 736}{154.2} = 95.45 \text{ mm.}$$

That is the mercury in the barometer must be raised to 736—95.45 640.55 mm.—above the level in the reservoir (*R*).

When the mercury has reached this level, or one near it, clip *3* is closed and the height at which the mercury stands very accurately noted. Clip *2* is then closed, after which the mercury is allowed to fall to zero by opening *3*. The tonometer is now removed and rotated so that the blood becomes spread out as a thin film on the walls, after which it is placed in a water-bath kept at about 40° C. in which it is constantly rotated for about 15 minutes.

On removal from the bath the pressure in the tonometer must again be measured. For this purpose the tonometer is reattached to *A* and the pump is turned on (with *3* closed) until the mercury has risen to the level at which it previously stood. Clip *4* is closed and *2* opened. If there has been no leak, and time has been allowed for the tonometer to cool down, there will be practically no difference between the two readings. If a difference of more than 5 mm. is observed it must be noted and the pressure prevailing in the tonometer taken as the average between the two readings.

Meanwhile 3 c.c. of freshly prepared weak ammonia water containing a trace of saponin (0.5 c.c. aq. ammonia in 500 c.c. water) has been placed in the blood gas bottle *B*. A pointed glass tube about 30 mm. long is now attached to the rubber tubing of the tonometer and this is removed from the barometer and held in vertical position above the bottle. The screw clip *2* is opened so that the air enters the tonometer, the clip *1*, is then cautiously opened to let a drop or two of blood flow out from the tip of the glass tube,* and after closing it again the end of the tube is wiped free of blood and placed in the bottle so that it dips under the ammonia solution. Clip *1* is now cautiously opened and about 1 c.c. of blood allowed to flow under the ammonia water. If this is done carefully the blood does not mix with the ammonia water which floats on the top of it as a layer and so prevents any diffusion of oxygen between the blood and the air. The bottle is firmly closed by its stopper, the pressure adjuster being meanwhile held open so that the level of the clove oil in the manometer is not disturbed. The bottle must now be submerged in a water-bath containing water at about room temperature, in which it is left until, with the adjuster closed, no further contraction of volume, due to cooling, is observed to occur.

The bottle is now removed from the bath and vigorously shaken so that the blood becomes laked and absorbs $O_2$ from the atmosphere of the bottle. After replacing the bottle in the bath and allowing time for cooling the difference between the levels of clove oil in the two limbs of the manometer is noted. With the adjuster open to the outside the stopper is removed from the bottle and about 0.25 c.c. of a freshly prepared saturated solution of potassium ferricyanide is placed in the small flat-bottomed test tube which is then lowered by means of a forceps into the fluid in the bottle, without allowing any of the ferricyanide to mix with the laked blood. After reinserting the stopper and cooling, the bottle is again removed from the bath and shaken so that the ferricyanide by mixing with the laked blood drives off the loosely combined oxygen and raises the pressure, which is measured by the manometer.

The relative amounts of reduced and oxy-hemoglobin present in the blood are proportional to the first and second readings of the manometer; when all is reduced hemoglobin the diminished pressure (shrinkage) recorded in the first

*Enough blood should be run out to bring the meniscus of blood in the tonometer to the upper file mark.

shaking of the bottle is practically the same as the increased pressure recorded in the second. They will not be exactly the same, since the volumes of bottle and tubing in the two cases are not the same, but the error thus incurred is inconsequential for most purposes (cf. Boyle's law).

The calculation of the percentage saturation of hemoglobin with oxygen is made by subtracting the first reading from the second, dividing by the second reading and multiplying by 100. Suppose in the observation made at 20 mm. partial pressure of $O_2$ the first reading is 24 mm. and the second, 108, then

$$\frac{108-24}{108} \times 100 = 77.7\% \text{ HbO and } 22.3\% \text{ Hb.}$$

The result must now be plotted on coordinate paper with the percentages of HbO along the ordinates and the partial pressures of oxygen on the abscissæ.

The observation is repeated at different pressures and by joining the points, the dissociation curve for blood is obtained. Care must be taken to see that the bottle is sufficiently shaken so that the partly reduced blood absorbs all the oxygen and gives it up again with ferricyanide. It is particularly in the latter operation that care must be taken.

*The influence of carbon dioxide in lowering the dissociation curve* can be readily shown by the method. The procedure is as follows: After the pressure has been reduced to the desired degree in the tonometer, the latter is placed in a horizontal position so that the blood lies along the walls, free of the ends. A $CO_2$ generating apparatus (Kipp's) or a bottle containing this gas is then connected by suitable tubing with the free end of the tonometer, care being taken before making the connection, to fill the tubing with $CO_2$. To accomplish this a slow stream of the gas is maintained and the air in the tubing beyond the screw clip (*1*) is squeezed out before connecting with the $CO_2$ generator. The most suitable partial pressure of $CO_2$ to work with is 40 mm. which is secured by cautiously opening screw clip *1* until with clip *2* open, but *3* and *4* closed, the mercury descends through 40 mm. in the barometer. Clips *1* and *2* are then tightly screwed down, and the tonometer removed, the further procedure being exactly as described above.

The effect of the 40 mm. of $CO_2$ will be found in the above example where a partial pressure of 20 mm. $O_2$ was used to reduce the percentage of HbO from 77 to about 35.

### THE $CO_2$-COMBINING POWER OF THE ALKALINE RESERVE OF THE BLOOD

After completing the estimations necessary for finding the percentage of oxyhemoglobin, in the experiments in which $CO_2$ is present in the tonometer, it is of interest to determine the amount of this gas with which the blood has combined. This will represent its ability to act as a buffer towards foreign acids. To perform the estimation it is necessary, however, to measure accurately the amount of blood which is removed from the tonometer to the blood gas bottle. This can readily be done by attaching a 1 c.c. pipette to the tubing of the tonometer (beyond clip *1*), a few drops of blood being allowed to escape from the pipette before delivering under the ammonia solution in the bottle, and precautions being taken not to take any of the upper layers of blood that had been exposed to full

atmospheric pressure when the tonometer was opened. This is done by removing the pipette from the tonometer before all the blood has run out.

To dislodge the $CO_2$ from the blood, the stopper is removed with the usual precautions and about 0.25 c.c. of a saturated solution of tartaric acid placed in the small test tube. After closing and allowing for temperature changes, the acid is shaken with the mixture of blood and ferricyanide, and the $CO_2$ thereby evolved measured by multiplying the displacement of the fluid in the manometer by a figure (the constant of the apparatus) obtained by a preliminary experiment in which a known amount of a standard carbonate solution is similarly treated.

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## AN APPARATUS FOR VOLUMETRIC SODIUM HYDRATE*

By Ralph R. Mellon, M.Sc., M.D., Dr. P.H., Rochester, N. Y.

THE variability of volumetric NaOH solutions as a result of the ease with which chemical combination is effected with $CO_2$ of the air is too common an experience to require comment. Accordingly I have devised an apparatus with which it is possible to keep such a solution indefinitely. The principle, exceedingly simple, makes use of the difficulty just mentioned, by freeing all air that comes in contact with the solution by previous contact with a concentrated solution of the alkali. Additional provision may be made for drying the same air with $CaCl_2$.

Given a bottle with outlet at the base, a burette stand and properly constructed burettes, anyone with a rudimentary knowledge of glass-blowing can construct the apparatus as above represented in a short time. The larger bottle (*A*) contains the volumetric solution, and the smaller one (*B*), the concentrated solution (10 per cent) of NaOH. *B* has a two-holed rubber stopper, one hole being occupied by a U-tube (c), the inner end of which is immersed in the solution, while the outer end permits the entrance of air to be freed from $CO_2$ in its passage through the 10 per cent NaOH. The other hole of the bottle is occupied by a straight piece of glass tubing, the inner end of which is not in contact with the solution, while the outer end leads to a filled $CaCl_2$ tube, by way of a flexible rubber tube (*d*). A gas-washing bottle of any convenient size could, of course, be used.†

The large bottle (*A*) has a one-holed rubber stopper which admits a glass (*T*), the lateral branch of which anastomoses with the $CaCl_2$ tube; the other

*From the Department of Laboratories, Hahnemann Hospital, Rochester, N. Y.

†A very helpful criticism of this apparatus has been made by a friend who has used a similar type. His experience has occasionally been that the expansion of the air above the solution forces the concentrated NaOH out of the U-tube (*C*). I have never had this experience, although, as he says, my apparatus may not be completely air-tight; yet the contained solution has never shown visible precipitate in the course of a year. In his own apparatus he is using a simple device remedying this defect, but I do not feel at liberty to appropriate it.

branch, after being bent at right angles, leads to the discharging burette (*E*). Of the two openings at the bottom of the latter, one is an outlet provided with a glass stopcock (*s*), the other an inlet similarly equipped, which receives the solution from the outlet (*o*) at the base of bottle *A*. These openings are connected by a piece of rubber tubing wired on at either end, and provided with a clamp as an additional precaution. The upper end of burette *E* admits a one-holed rubber stopper, which in turn gives passage to a T-shaped glass tube (*F*). Both branches of the *T* outside the burette are bent at right angles, one connecting with the free arm of the glass *T* in Bottle *A* by rubber tubing, the other attached to a piece of rubber tubing kept tightly closed with a Hoffman clamp. Burette *E* is held by clamp of stand *H*, in which I find it convenient to have an additional empty burette.

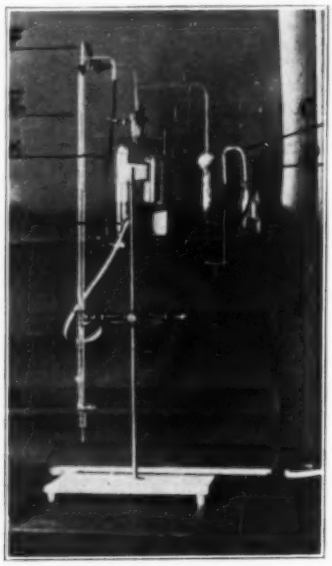

Fig. 1.

The apparatus is wholly portable. The flexible rubber tube (*d*) in bottle *B* makes it possible to hold both bottles in the right hand, while the standard of the burette stand may be grasped with the left hand. This is of advantage, especially when it is necessary to replace the $CO_2$ containing air above the solution and in the connecting tubes with $CO_2$ free air. This is only necessary when the NaOH solution is first put in bottle *A*. At this time the apparatus can be carried to the aspirator, to which it is connected by tube (*k*). The aspiration is continued until the air from *k* reacts negatively with $BaCl_2$. This test is made by attaching temporarily a rubber tube to U-tube *C*, immersing tube *k* in $BaCl_2$ solution, into which the contained air is forced by blowing into U-tube (*c*). The glass connecting tubes, including the one containing the $CaCl_2$, we readily made with a blast lamp.

# TECHNIC FOR THE WASSERMANN REACTION*

By John H. Larkin, M.D., Leon H. Cornwall, M.D., and I. J. Levy, M.D.,
New York City

THE following technic is employed at the City Hospital Pathological Laboratories in performing the Wassermann reactions. It is, in its essential details, the Wassermann-Citron technic, with certain modifications in accordance with the more recent research advances in this field.

### VOLUME

The total volume of the reactions is 2.5 c.c., being one-half the amount of the original volume used by Wassermann and his coworkers. Each ingredient is diluted, in accordance with preliminary titrations, so that for the test .5 c.c. of each ingredient is added. The figures given below apply to the original Wassermann amounts, and for their application to our work are divided by two.

### PREPARATION OF SERA

Blood sera are sent to the laboratory on the afternoon of the day previous to that on which tests are performed. They are allowed to coagulate and stand at room temperature from one-half to two hours. The clots are then carefully separated from the sides of the tubes with a fine sterile wire and the tubes placed in the ice chest overnight. On the following morning the sera are poured off or pipetted from the clots, centrifugalization being resorted to only in cases where the sera are not clear and free from cells.

All sera are inactivated in a water-bath at 55° C. for one-half hour.

### HEMOLYTIC SYSTEM

The hemolytic system consists of sheep cells and antisheep hemolytic amboceptor derived from rabbits.

### CORPUSCLES

Corpuscles are obtained fresh from an abattoir on the day previous to that on which the tests are performed. The whole blood is collected in a sterile glass bottle, tightly stoppered and containing glass beads. It is defibrinated by gentle shaking for fifteen to thirty minutes. After defibrination it is filtered through twelve thicknesses of sterile gauze to remove any small clots of fibrin including, in their meshes, cells. The filtrate is placed in 15 c.c. centrifuge tubes and centrifuged four times at high speed. The first centrifugalization is for 25-30 minutes. The pure serum is then pipetted off and the cells well mixed with .9 per cent sterile saline. The second, third and fourth centrifugalizations are for 25, 15 and 15 minutes respectively, the saline being pipetted off and fresh saline added each time. Samples of blood showing any hemoglobin tinge in the super-

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natant saline after the third or fourth centrifugalization are discarded. The washed corpuscles are placed in the ice chest overnight, and on the following morning a 5 per cent suspension of the packed corpuscles is made up for titrations and tests.

### COMPLEMENT

Guinea pig complement is obtained by bleeding to death from the carotid artery from three to five, preferably the latter, animals. Average-sized animals, 250 to 500 grams, are selected when this is possible, and they are not fed for at least twelve hours before the bleeding. Unnecessary excitement of the animals is avoided. The complement is obtained in the late afternoon preceding the day of the tests. It is allowed to coagulate at room temperature; the clot is separated with a small sterile wire, and the containers (15 c.c. centrifuge tubes) are placed in the ice chest overnight. On the following morning the serum is pipetted off and centrifuged once at high speed, until all cells are thrown down. It is then pipetted to another sterile container and placed in the ice chest until used. This usually yields a clear, slightly yellowish serum. A slight hemoglobin tinge is of no particular importance.

### AMBOCEPTOR

The antisheep hemolytic amboceptor is placed in small, sealed sterile ampules after inactivation at 56° C. for one-half hour. Each ampule contains approximately 5 c.c., and they are kept in the ice chest near the ice.

### PRELIMINARY TITRATION OF HEMOLYTIC SYSTEM

*Amboceptor.*—Each batch of amboceptor is tested for its hemolytic titer with 1 c.c. of 1:10 dilution of complement from 3-5 guinea pigs and 1 c.c. of 5 per cent sheep cell suspension. This titration is repeated on four or five occasions with different batches of complement and the average minimal amount

TABLE I

TITRATION OF ANTISHEEP HEMOLYTIC AMBOCEPTOR—RABBIT IMMUNE SERUM

| TUBE NO. | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Amboceptor 1:500 | 0.5 | 0.45 | 0.4 | 0.35 | 0.3 | 0.25 | 0.2 | 0.15 | 2.0 | 0.0 | 0.0 |
| Saline .9% | 0.5 | .55 | 0.6 | 0.65 | 0.7 | 0.75 | 0.8 | 0.85 | 0.0 | 0.0 | 0.0 |
| Complement 1:10 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 0.0 | 1.0 | 0.0 |
| Saline .9% | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 | 3.0 | 4.0 |
| Cells, 5% emulsion | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| Incubation, waterbath, 1 hr., 37.5° C. | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... |
| Resulting hemolysis | C | C | C | C? | marked | med. | sl. | 0 | 0 | 0 | 0 |

which causes complete hemolysis (perfectely clear red solution with no turbidity or sedimented cells) at the end of one hour in the water-bath at 37.5° C. is recorded as the unit titer for that amboceptor. Before each group of reactions the titration of the amboceptor is repeated with the pooled complement of that day as a check. Table I illustrates the order used for the addition of the ingredients

for the titration of the amboceptor before every series of tests. For the reaction two units are always employed.

*Complement.*—Each batch of pooled complement is titrated against an excess (2 units) of amboceptor, the unit of amboceptor having been previously determined, as described above. One unit of complement is that amount which produces complete hemolysis (perfectly clear red solution with no turbidity or sedimented cells) at the end of one hour in the water-bath at 37.5° C. Table II illustrates the order used for the addition of the ingredients in the complement titration. For the reaction two units are employed.

One actual unit of amboceptor on the basis of the above titration lies between .35 and .4 c.c. of the dilution of amboceptor employed (1:500). For practical purposes .35 might safely be used, but 4 is taken to be the full unit, and the amboceptor is diluted 1-625 and 1 c.c. of that dilution added for the test, this being the equivalent of .8 c.c. of 1-500 dilution or two full units. As stated, for practical purposes, .35 c.c. of the 1:500 dilution is one unit and .7 c.c. two units, so that the 1 c.c. of the 1:625 dilution, employed in the reaction, represents practically three units.

The method given below for the readings of the degree of hemolysis will illustrate that complete hemolysis is that degree which results in a perfectly clear, red solution with no turbidity or sedimented cells at the end of the prescribed period of one hour. Slight differences occur, if identical titrations are done in duplicate at the same time, by the same person and with the same reagents. In other words, a certain latitude must be allowed for the unavoidable error in the standardization of pipettes and for the human error that can not be compensated for. The same remarks apply to the complement titration shown in Table II.

All amboceptor dilutions are made from one original dilution of 1:1000, a sufficient amount of this being made for the day's work.

All complement dilutions for titrations are made from one original dilution of the pooled complement of 1:5.

So far as feasible, the same pipette is used for diluting the amboceptor and for adding it to the several tubes. The same precaution is also taken in adding the complement to the tubes of the complement titration.

Saline from one flask containing a sufficient amount for one day's work is employed for all titrations and tests. So far as feasible, but one pipette is used for saline.

TABLE II

TITRATION OF COMPLEMENT

| TUBE NO. | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Complement 1-10 | 0.8 | 0.6 | 0.5 | 0.45 | 0.4 | 0.35 | 0.3 | 0.25 | 1.0 | 2.0 | 3.0 |
| Saline .9% | 0.2 | 0.4 | 0.5 | 0.55 | 0.6 | 0.65 | 0.7 | 0.75 | 0.0 | 0.0 | 0.0 |
| Amboceptor 1-625 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 0.0 | 0.0 | 0.0 |
| Saline .9% | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 | 3.0 | 2.0 | 1.0 |
| Cells 5% Emulsion | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| Incubation water-bath 1 hr. at 37.5° C. | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... |
| Resulting hemolysis | C | C | C | C | C? | marked | med. | sl. | 0 | 0 | med. |

With two full units of amboceptor as determined by the titration in Table I one full unit of complement equals .45 c.c. of a 1:10 dilution. For the reaction .9 c.c. of a 1:10 dilution is employed. The actual unit of complement as determined from the above titration lies between .4 and .45 c.c. We do not consider that a greater number of dilutions are feasible for practical work. From a practical standpoint .4 c.c. of the 1:10 dilution represents one unit and .9 c.c. approximately 2¼ units.

Ingredients are added in the order shown in the tables above. After the addition of each ingredient, the mixture is thoroughly shaken. For the test each ingredient is made up in such dilution that 1 c.c. of each is added, with the exception of the complement. During the incubation of the titrations and tests the tubes are shaken at fifteen-minute intervals.

Controls are made for the following:

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Isohemolytic | activity | of | amboceptor in excess (5 units) | Tube | 9, | Table | I |
| " | " | " | saline | " | 11, | " | I |
| " | " | " | complement | " | 10, | " | I |
| " | " | " | " | " | 9, | " | II |
| " | " | " | " | " | 10, | " | II |
| " | " | " | " | " | 11, | " | II |

Complements which show any more than a slight isohemolytic activity in an amount slightly in excess (.2 c.c. of 1:10 dilution) of the amount that represents two units or that are of very weak hemolytic activity (.8 c.c. of 1:10 dilutions) with the excess of amboceptor are discarded.

The following method is used for readings of hemolysis:

| | | | |
|---|---|---|---|
| Clear, red, no turbidity or sedimented cells—complete | .... or 4-plus | hemolysis | (100%) |
| Slight turbidity, but no cells—complete? | .... or 4-plus? | " | (100%?) |
| Turbidity with few (25%) cells—marked | .... or 3-plus | " | (75%) |
| 50% sedimented cells—moderate | .... or 2-plus | " | (50%) |
| 75% " " —slight | .... or 1-plus | " | (25%) |
| 100% " " —none | .... or 0 | " | |

## ANTIGENS

Three antigens are employed routinely: (1) a cholesterin reinforced antigen, either human or guinea pig heart; (2) an alcoholic extract of beef, guinea pig or human heart; (3) an alcoholic extract or Noguchi acetone-insoluble lipoidal antigen.

As is well known, antigens made at different times and by different methods or with different tissues vary greatly in their fixing property. It is usually true in our work that we have antigens with varying degrees of fixing power. The first antigen is always the strongest and the second and third respectively, weaker. In this manner we can estimate the relative strength of positive reactions. New antigens are never used for routine work until their comparative strengths have been tested out with old antigens of known strength, with from two to five hundred different blood sera.

## METHOD OF ANTIGEN PREPARATION

*Guinea Pig Heart.*—1. Hearts are removed and dissected free from fat and other extraneous tissue. They are washed free from blood in .9 per cent saline.

2. Washed hearts are placed in absolute alcohol and kept at room temperature, until approximately 100 have been collected.

3. Alcohol is poured off; hearts are finely minced; dried and weighed.

4. Absolute alcohol is added in the proportion of ten volumes to one volume of heart tissue, using the alcohol in which the hearts were originally collected.

5. The alcoholic extract is placed in the thermostat at 37.5° C. for 14-21 days, being shaken at least once daily.

6. The alcoholic solution is filtrated off and the antigen titrated for its antigenic, anticomplementary and hemolytic doses.

Alcoholic extracts of human and beef hearts are prepared in the same manner as those of guinea pig hearts, with the omission of step two and the first part of step three. Acetone-insoluble, ether-soluble lipoidal antigens are prepared in accordance with the method outlined by Noguchi.

Alcoholic extracts are reenforced by the addition of .4 per cent Kahlbaum's cholesterin. Small quantities of alcoholic extracts are reenforced with cholesterin at frequent intervals, so that they are used up within from three to four weeks.

### ANTIGEN TITRATIONS

All antigens are tested for the minimal antigenic, anticomplementary, and hemolytic doses. It is not considered necessary here to detail the protocols for these titrations. With cholesterinized antigens the dose employed in the test is from five to twenty times the minimal antigen dose, or that dose producing complete inhibition with several (3-5) pooled positive sera. The precaution is taken to select sera that are not anticomplementary in double the amount used for the test (.4 c.c.), and that have no natural antisheep amboceptor. No more than one-fifth the anticomplementary or hemolytic dose of cholesterinized antigens is ever used. The latter factor seldom renders any of our antigen useless, but the anticomplementary feature is of the utmost importance.

An average titration of our cholesterin-reenforced guinea pig heart and human heart antigens is represented as follows:

| | |
|---|---|
| Minimal antigenic dose (with pooled positive sera) | 0.005 c.c. of undiluted antigen |
| Anticomplementary dose | 0.35 " " " " |
| Hemolytic dose | 0.45 " " " " |

For the reaction 1 c.c. of a 1:20 dilution (the equivalent of .05 c.c. of the undiluted antigen) is employed.

In the case of simple alcoholic extracts and Noguchi antigens the dose in the test represents two or three times the minimal antigenic dose, and never more than one-half the minimal anticomplementary dose. In practice our antigens have such wide differences between the antigenic and anticomplementary doses that the limits are much greater than this.

The order for the addition of ingredients in the titrations of antigen is as follows:

For antigenic dose:

| | |
|---|---|
| Antigen, plus saline, | 1 c.c. |
| Pooled positive serum, plus saline, | 1 c.c. |
| Complement, plus saline, (2 units) | 1 c.c. |
| Incubation water-bath 1 hr. at 37.5° C. | |
| Cell suspension 5 per cent<br>Amboceptor (2 units)<br>Sensitized for ½ hr.<br>in water-bath | 2 c.c. |

For anticomplementary dose the same with the omission of positive serum, the total volume being made to 5 c.c. with saline.

For hemolytic dose the same with the omission of positive serum and amboceptor, the total volume being made to 5 c.c. with saline.

REACTION

1. The original Citron method of using two tubes for the reaction proper, the second tube containing one-half the dose of antigen and serum used in the first tube has been followed for the first two antigens. With the third antigen only one tube with the full dose of antigen and serum is used. Each serum is controlled for its anticomplementary property with double the amount used in the test proper.

2. Owing to the necessity of conserving reagents, the Citron method has been modified to the extent of omitting the second tube in all recent work.

Metal racks with two rows are used, and the tubes are set up as follows:

*Method I*

1. Citron method for first two antigens; single tube for third.

| | Front row | | | Back row | | |
|---|---|---|---|---|---|---|
| Tube | 1 | 2 | 3 | 1 | 2 | 3 |
| Tube No. | | | | | | |
| Antigen | 1.0 c.c. | 1.0 | 1.0 | 0.5 | 0.5 | 0.0 |
| Saline | 0.0 | 0.0 | 0.0 | 0.5 | 0.5 | 1.0 |
| Serum | 0.2 | 0.2 | 0.2 | 0.1 | 0.1 | 0.4 |
| Saline | 0.8 | 0.8 | 0.8 | 0.9 | 0.9 | 0.6 |
| Complement 1:10 | 0.9 | 0.9 | 0.9 | 0.9 | 0.9 | 0.9 |
| Saline | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |

Incubation for 1 hour in water-bath at 37.5° C.

Cell suspension 5% Amboceptor diluted so that 1 c.c. equals 2 full units. } Equal parts of cell suspension and amboceptor sensitized in thermostat for one-half hour 2 c.c. to each tube.

Incubation until controls completely hemolyze; usually 20 to 30 minutes.
First reading immediately.
Second reading after standing overnight at room or ice box temperature.

2. Method identical with that detailed above, with the exception of the omission of tubes 1 and 2 in back row, two antigens being placed in the front row; viz., a cholesterin and alcoholic extract; one antigen placed in first tube of back row; viz., another alcoholic extract or a Noguchi antigen; serum control placed in second tube of back row.

### METHODS OF READING

With the Method I the Citron method of degree reading is used for the first two antigens (Antigen 1 cholesterinized—Antigen 2 strongest noncholesterinized antigen, either sample alcoholic extract or Noguchi), and merely a positive or negative reading on third antigen.

With Method II the Citron method has been modified so that the weaker antigen used in the second tube is considered as though it were the same as the half amount of antigen and serum tube (back row 1 and 2) of the original method.

### INTERPRETATION OF WASSERMANN READINGS

The readings of antigen No. 3 (See Method I) are omitted because they are always weaker than those of the second antigen. Many of the combinations shown above occur seldom, or never, the second antigen in practically every case giving complete hemolysis in the reaction before there is any hemolysis shown with the cholesterinized antigen (No. 1).

METHOD I

| ANTIGEN NO. 1 CHOLESTERINIZED | ANTIGEN NO. 2 NEXT WEAKER ANTIGEN | ANTIGEN NO. 3 SECOND WEAKER ANTIGEN | INTERPRETATION |
|---|---|---|---|
| | 4-plus | | Very strongly positive |
| 4-plus | 3-plus | | " " " |
| 4-plus | 2-plus | | " " " |
| 4-plus | 1-plus | | Strongly positive |
| 4-plus | Doubtful | | " " |
| 3-plus | 3-plus | | " " |
| 3-plus | 2-plus | | " " |
| 3-plus | 1-plus | | " " |
| 3-plus | Doubtful | | " " |
| 4-plus | Negative | | Positive |
| 3-plus | Negative | | " |
| 2-plus | 2-plus | | |
| 2-plus | 1-plus | | Weakly positive |
| 2-plus | Doubtful | | " " |
| 2-plus | Negative | | " " |
| 1-plus | 1-plus | | Very weakly positive |
| 1-plus | Doubtful | | " " " |
| 1-plus | Negative | | " " " |
| Doubtful | Doubtful | | Doubtful |
| Doubtful | Negative | | " |
| Negative | Negative | | Negative |

METHOD II

| ANTIGEN NO. 1 CHOLESTERINIZED (ONE TUBE) | ANTIGEN NO. 2 NEXT WEAKER ANTIGEN (ONE TUBE) | INTERPRETATION |
|---|---|---|
| 2-plus | 2-plus | 4-plus .... Very strongly positive |
| 2-plus | 1-plus | 3-plus .... Strongly positive |
| 2-plus | Doubtful | 2-plus .... Positive |
| 2-plus | Negative | 2-plus .... " |
| 1-plus | Doubtful | 1-plus .... Weakly positive |
| 1-plus | Negative | 1-plus .... Very weakly positive |
| Doubtful | Negative | ? .... Doubtful |
| Negative | Negative | – .... Negative |

CONTROLS EMPLOYED IN TITRATION

1. Amboceptor for isohemolytic activity.
2. Complement for isohemolytic activity.
3. Saline for isohemolytic activity.

CONTROLS EMPLOYED IN REACTION

1. Each serum for anticomplementary property in double amount used in test.
2. Positive luetic serum.
3. Negative or normal serum.
4. Cholesterin antigen for anticomplementary property in five times amount used in test.
5. Simple alcoholic extracts and Noguchi antigens in double or triple amounts used in test.

In order to completely duplicate in preliminary titrations the conditions of the actual Wassermann test, two procedures are now followed which are not indicated in the description and protocols above. Complement is placed in the incubator for one hour before its use in titrations of the hemolytic system, and the amboceptor and cell suspensions are sensitized for one-half hour before hemolytic titrations.

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## A METHOD FOR PRESERVING A SHARP SCALPEL IN EXPERIMENTAL SURGERY*

By F. C. Mann, M.D., Rochester, Minn.

ONE of the problems in the mechanics of surgery is to maintain instruments in good working condition. In experimental surgery it has been found difficult to keep a good edge on scalpels, mainly due to the fact that section of the animals' skin dulls the edge of the knife. This problem is solved by obtaining a cheap knife to make all skin incisions. A high grade, all metal, small paring knife has been found to suit our purpose. These knives are made in various shapes so that almost any style can be obtained. They sharpen readily and are quite strong. As they are composed entirely of metal they withstand boiling. They usually can be used three or four times before they need to be retouched on the stone.

The present technic for making an incision is as follows: After the usual preparation, the skin and fascia are cut with one stroke of the paring knife which we term the "skin knife." The sharp, heavy blade of the knife makes a sure, clean incision possible. As soon as the skin incision is made, the knife is dropped in the pan for used instruments and after catching the bleeding points and placing the skin towels in position the rest of the incision is made with the regular scalpel. While the knife was obtained for the purpose of saving the edges of the scalpels, it also makes possible another point of technic which some surgeons hold important, namely, the use of a scalpel which has not passed through the skin for the deep part of the incision.

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## EDITORIALS

### *The Negro Population*

THE Census Bureau has issued a most interesting volume on the negro population of this country from 1790 to 1915, a period of 125 years. At the beginning of this period there were 757,208 negroes in this country with 239 for every 1000 of the white population. Up to 1808 the negroes were increased by importation, but the proportion to the whites has constantly decreased. Since 1910 neither immigration nor emigration has been great enough among negroes to materially affect the number and the increase has been wholly due to native births. In 1910 the number of negroes was 9,827,763 and for every 1000 whites there were 120 negroes. The percentage of increase in the succeeding decennial periods has gradually fallen from 30.5 in 1830 to 11.2 in 1910. Although the percentage increase fell off from period to period the lower percentages for the later periods represent larger absolute additions to the population.

In 1790 the negro population of Virginia numbered 305,493, making two-fifths of the total in the United States. In the same year the negroes in New York numbered 25,978, this state being sixth in the list. In 1860 Virginia still

had the lead with a negro population of 548,907 and was followed in order by Georgia, Alabama, Mississippi, and South Carolina, each of which had more than 400,000. In the census of 1870 and in each succeeding census Georgia has stood at the head of the list with a negro population in 1910 of 1,176,987. In the South as a whole 29.8 per cent of the population in 1910 was negro; in the North 1.8 per cent, and in the West 0.7 per cent. The negro proportion was highest in the South Atlantic division in which it reached 33.7 per cent. In the East South Central it was 31.5 per cent, and in West South Central 22.6 per cent. In the North the negro proportion in the population has not changed materially since 1850, and it has not at any census since that date amounted to so much as 2 per cent. In the North the percentage declined from 3.4 in 1790 to 2 in 1850. At no census has the proportion in the West amounted to more than a fraction of one per cent. Washington had in 1910 the largest negro population, while New York came second. Birmingham had the largest increase in negro population during the decade 1900-1910, with New York second. Taking the country at large, the negro is the most rural. Only 27.4 per cent of the negroes were in 1910 urban, while among native whites born of natives, the urban percentage was 36.1; of mixed parentage 59.4 per cent; of double foreign parentage 68.1 per cent; of foreign born whites 72.2 per cent. These figures indicate that the foreign element tends to possess our cities and exclude both the native white and the negro. In 1910 the negro constituted 6.3 per cent of the urban population of the United States, 28 per cent of that of the South, 2.4 per cent of that of the North and 1 per cent of that of the West. When the negro migrates from the South to the North he generally goes to a city, but the percentage of the negro urban population of the North did not increase from 1890 to 1910. This does not mean that negroes did not come from the South to the North during this twenty-year period, but such a movement was neutralized when calculated in percentage by increased white urban population in the North. In the South as a whole the percentage of negroes in the urban population decreased from 31.7 per cent in 1890 to 28 per cent in 1910, and in the rural population from 34.2 per cent in 1890 to 30.3 in 1910. There are thirty-two counties in the South which report no negroes, twenty-eight of these are in Texas, two in Oklahoma, and one each in Arkansas and North Carolina. The District of Columbia, regarded as a county, contains the greatest number of negroes; Shelby County, Tennessee, including Memphis, comes next, and Jefferson County, Ala., including Birmingham, stands third. In 1910 there were males of military age (18-44 years) 20,473,684, of which 1,985,415 were negroes and 18,351,870 whites; the latter being divided into native whites, 13,880,182 and foreign born 4,471,688.

In the period of sixty years from 1850 to 1910 the proportion of mulatto in the negro population increased from 11.2 to 20.9 per cent. In 1850 the proportion of mulatto in the free colored population greatly exceeded the proportion in the slave population, the number of mulattoes to 1000 blacks being 581 in the free and 83 in the slave population. The number of mulattoes to 1000 blacks in 1910 was 252 in the South, 363 in the North, and 473 in the West. While in round numbers one-third of the blacks are illiterate, only one-fourth of the mulattoes are unable to read and write. May we infer from these facts that the negro is to be absorbed by the white race? It seems that cohabitation is more

common now than in slave times and that the mixed race is being better educated than the original.

There are some interesting points concerning the marital state. The negro, both male and female, both urban and rural, marries at an earlier age than the white; though taking all ages, about the same per cent of the males of both races marry, while more white women than negroes marry. Widowhood is much higher among negro than among white women. There are also more divorces among negroes than whites. The tendency of negroes to marry at younger ages than the whites is clearly in evidence in the statistics for both urban and rurals. In the urban population 31.6 per cent of negro males aged 20 to 24 years as compared with 14.8 per cent of native white males of this age were married, the corresponding percentages for rural communities being 40.4 for negroes and 26.3 for native whites. Among females 20 to 24 years of age in urban communities the percentage married was 50.5 for negroes and 38.7 for native whites; the corresponding percentages in rural communities being 63.2 and 55.6. In the South among both male and female negroes the percentage single is highest in cities of 100,000 and over and lowest in the rural population.

In 1820, although the proportion of children under 14 in the negro population was approximately the same as the proportion in these ages in the white population, the number of such children per 1000 women, 14-44 years of age, in the negro population was considerably below the number in the white population. In 1910 both the proportion of children in the negro population and the proportion of children to women very considerably exceeded the corresponding proportions among whites. In both races the decline for the proportion of children under 14 for the period of 90 years from 1820 to 1910 is very marked. Among the negroes it is somewhat less than it is among whites, although it is to be noted that in the latter decades of this period for which data are available in greater detail the decrease in the number of children under five years of age per 1000 women 15 to 44 is in the aggregate somewhat greater among negroes than among whites, the result being a closer approximation of the ratio for negroes to that of whites.

It is impossible to obtain exact data on the annual death rate in the negro population. At each decennial census from 1850 to 1900 there was an attempt made to collect mortality statistics by inquiring of the living concerning those who had died during the twelve months of the census year. From the first this method of gathering mortality statistics was recognized as an absolute failure. It was a demonstration of the fact that the dead are easily and quickly forgotten. The compilers of the census of 1850 recognizing this wrote, "In regard to the number of deaths, the returns of the census are not likely to deceive anyone, since an attempt to reason from them would exhibit a degree of vitality and healthfulness in the United States unparalleled in the annals of any nation. * * * * Nothing short of a registration system in the States can give the required data satisfactorily since people will not, or can not, remember and report to the census taker the number of facts and the particulars which occur in the period of a whole year or longer prior to the time of his calling."

The states and cities are now building up the registration of births and deaths and the census maker relies on these and has ceased to trust the memory

of the living concerning either the names or numbers of the dead. Now (1919) the registration area embraces about 70 per cent of our population and covers about 50 per cent of our area, but the mass of the negro population is not in this area. Most cities in the South belong to the registration area and the states are gradually but slowly progressing in this direction. All the census maker can do is to take the annual death among negroes and whites in the registration area. In 1910 the annual death rate among all whites in this country was 14.6 per 1000, while the corresponding figure for the negro population was 25.7. In 1910 there were only four countries in the world keeping any kind of mortality statistics with a higher annual death rate than that prevalent among the negroes in our registration area. These were Ceylon (27.3) European Russia (28.9) Chile (31.1) and Mexico (33.3).

Is this high death rate among our negroes due to racial defects or to environmental conditions? Upon the true answer to this question the future of the race largely depends. Many European countries now with an annual death rate comparable with that among our whites had a generation or more ago a rate quite as high as that among our negroes now. In 1910 the death rate in Holland was 13.6; in 1860, it was 25.4. That of Germany in 1910 was 16.2; in 1885 it was 25.1. It seems therefore if the high death rate among the negro population is wholly due to environment we may expect a great improvement within a few generations.

"Although negro infant mortality in 1910 was greatly in excess of white infant mortality—the excess of the negro over the white rate being 136.9 for males and 126.7 for females—the figures for 1910 indicate marked improvement during the decade 1900-1910. In this period infant mortality among negro males declined from 380 in 1900 to 280.3 in 1910, the decrease in the rate amounting to 99.7, and the rate for negro females declined from 309.7 in 1900 to 243.2 in 1910, the decrease amounting to 66.5." There could be no better evidence of increased intelligence among negro mothers.

"Three principal causes of death in the negro population, namely, tuberculosis, pneumonia and organic diseases of the heart—are the principal causes of death also for the white population. Per 1000 deaths from all causes in 1913, in the negro population, 366 or more than one third, and in the white population, 291 are classified under one or other of these three causes."

However, tuberculosis is much more prevalent in the negro population than in the white, being 176 to 98. In the white population pneumonia and organic diseases of the heart are nearly of equal importance with tuberculosis as causes of death, the number of deaths from these two causes per 1000 deaths from all causes being 94 to 99 respectively. In the negro population these causes are relatively to other causes much less prevalent than tuberculosis, the number per 1000 deaths from all causes in the negro population being for pneumonia 100 and for organic diseases of the heart 90.

"Tuberculosis developed a rate per 100,000 population in 1910 of 463 among negroes, which was more than three times the tuberculosis rate of 148 per 100,000 white population. The rate for pneumonia was 298 or more than twice the pneumonia rate of 142 among whites. The rate for organic diseases of the heart was 212 among negroes and 139 among whites." It appears from these

figures that each of these three diseases is either more prevalent or more fatal, or both more prevalent and more fatal among negroes than among whites. Is this due to greater susceptibility on the part of the negro or to living conditions? So far as pneumonia is concerned, the evidence gathered from the army camps indicates that there is not a marked difference between the southern white and the southern black and both are more susceptible to this disease than their comrades of either color from the North. Under ordinary conditions army statistics show that pneumonia is more deadly the farther South one goes. This was doubtful, however, with post influenza pneumonia.

We are inclined to advance a tentative opinion that the higher death rates from tuberculosis and pneumonia among the blacks are due to living conditions while the higher death rate from heart disease is due to the wider prevalence of syphilis among the blacks. However, this point needs more careful study.

The diseases more prevalent among whites are cancer— with an annual rate of 57 among negroes and 77 among whites per 100,000 population—diphtheria, measles, diabetes, and cirrhosis of the liver.

So far as school attendance is concerned, the writer of the report before us makes the following statement: "Reviewing the situation as a whole, while it is true that the proportion of negro children not in school is large, not only in rural communities but even in large cities; and although in the South, the proportion of negroes not in school greatly exceeds the corresponding proportion of whites, still the data for school attendance presented establishes the fact of rapid improvement in the condition of negroes in the South. * * * * Should the next census show a similar advance the negro race in its school attendance will not stand far behind the white."

Taking all inhabitants over ten years of age the percentage of illiteracy among negroes was in 1910, 30.4; among whites 5.0. Taking all between ten and fourteen, the figures for the blacks are 18.9, for the whites 1.8. In 1890, 39.8 per cent of the negro population ten to fourteen years of age were illiterate; in 1900, 30.1 per cent, and in 1910, 18.9 per cent. Of course the percentage of negroes who attain anything more than an elementary education remains small.

In criminality the negro makes a bad showing and he behaves far worse in the North than in the South. The following excerpts illustrate: "30.6 per cent of the total number of prisoners and juvenile delinquents enumerated on January 1, 1910, and 21.9 per cent of the total number committed during the year 1910 were negroes, whereas the percentage of negroes in the total population was only 10.7. Per 100,000 negro population the number of negro prisoners and juvenile delinquents enumerated on January 1st was 424.6 and the number committed during the year 1101.7, the ratios for negroes being markedly in excess of the corresponding ratios shown for whites. * * * * They represented 56 per cent of the total number committed for grave homicide, 49 per cent of the total for lesser homicide, and 41.1 per cent of the total for assault. They contributed 33.6 per cent, or one-third the commitments for prostitution and fornication, and almost as large a proportion of the commitments for robbery, burglary, and for violating liquor laws. On the other hand they comprised only about 16 per cent of those committed for drunkenness and disorderly conduct and for vagrancy." In the North the serious offenses committed by the negro are arranged in the

order of frequency as follows: (1) grave homicide, (2) prostitution and fornication, (3) lesser homicide, (4) robbery, (5) assault. In the South they are as follows: (1) assault, (2) grave homicide, (3) larceny, (4) robbery, (5) burglary. The ratio of commitments as between blacks and whites is from two to three times as high in the North as in the South. This means either that the southerner is more lenient with the negro or that the negro behaves better in the South.

The compilers of the report make statements concerning the relative prevalence of insanity in the two races with caution. The figures are based on the number admitted to and in asylums during the census year. "Negroes constituted 6.9 per cent of the insane enumerated at the beginning of the year and 7.2 per cent of the insane admitted during the year. The percentage of negro insane was accordingly very considerably below the percentage of negroes in the population (10.7). For negroes the ratio of inmates present on January 1st per 100,000 negro population was 131.4, the corresponding figures for whites being 213.2. The ratio of admission per 100,000 population was 44.6 for negroes and 68.7 for whites. These ratios did not, however, obtain with any degree of uniformity in the several geographical divisions and states. Outside the South the ratio for negroes was in fact higher than that of the whites in each division. In New England, for instance, the ratio per 100,000 population for inmates enumerated on January 1st was 473.6 among negroes and 296.9 among whites, the corresponding figures for admissions being 153.8 for negroes and 105.9 for whites."

The conclusion seemingly justified by these statements is that the negro in the North is more prone to insanity than his brother in the South, or the southern negro, though insane, is less frequently sent to asylum.

There are no trustworthy statistics concerning the feeble-minded.

The number returned as blind per 100,000 negro population was 90, the corresponding proportion for whites being 58.2 per 100,000. This is most certainly due to the greater prevalence of gonorrhea among the blacks.

"The figures rather confirm the supposition that deaf-mutism is less common among negroes than among whites." The compilers of the census attribute this to the lighter prevalence of measles, scarlet fever and diphtheria among negroes and say: "While the death rate from measles in 1914 was only slightly higher for whites than for negroes, the death rate from diphtheria was nearly twice as great and that for scarlet fever was practically four times as great for whites as for negroes. On the other hand, negroes appear to be somewhat more susceptible to meningitis, another leading cause of deaf-mutism, than are whites."

Counting as paupers those sent to and maintained in almshouses, the negro slightly outnumbers the native white in the South; in the North he doubles the native white; while in all sections of the country the foreign-born white exceeds the combined strength of his competitors.

In regard to the ownership of homes the census enumerators were instructed as follows: "A home is to be classed as owned if it is owned wholly or in part by the head of the family living in the house. It is not necessary that full payment of the property should have been made."

"Every house not owned either wholly or in part by the family living in it should be classed as rented whether rent is actually paid or not."

Owned homes are divided into free owned and mortgaged.

With the above understanding, owned homes increased from 18.7 per cent of all negro homes in 1890 to 21.7 per cent in 1900, to 23.3 per cent in 1910.

The percentage of free owned homes slightly decreased during the decade 1900 to 1910. "This decline was not due to any decrease in the number of free owned homes—the number owned free, both of farm houses and of other houses, increasing in this decade as in the decade following—but to a relatively more rapid increase in the number of homes owned under a mortgage or other lien. Such ownership is undoubtedly in many cases a first step out of tenancy."

"Of negro farm homes 98.5 per cent were in the South in 1910, 1.4 per cent in the North and 0.1 per cent in the West."

"The term 'family' as defined for census enumeration means a group of persons whether related by blood or not who live together as one household, usually sharing the same table."

As thus defined the negro family averaged exactly the same number as the whites in 1910 (4.5). This gives us no information concerning housing conditions. There may be several families in one house.

"In the negro population, both male and female, the proportion gainfully employed increased in each of the last two decades, among males from 79.4 per cent in 1890 to 84.1 in 1900, and to 87.4 per cent in 1910; and among females from 36.2 per cent in 1890 to 40.7 per cent in 1900, and to 54.7 per cent in 1910." Taking the whole negro population over ten years of age in the country 29 per cent were not gainfully employed in 1910. Of the male negro population ten years of age and over in the South, 56.6 per cent were engaged in agricultural and 31.1 per cent in nonagricultural pursuits, the percentages for females being 32.4 and 23.5. In the North and West 75.7 per cent of the negro males and 45.1 per cent of the females were engaged in nonagricultural pursuits; the proportion engaged in agricultural pursuits being 10.1 per cent for males and 0.4 per cent for females, and the proportion not gainfully employed 14.2 for males and 54.5 per cent for females.

More than one-third of the total negro women returned as gainfully employed in 1910 were returned in two occupational groups, as either "servants" or "laundresses not in laundries."

In 1910 negroes made up 14 per cent of all farmers in the country. In many of the mountain and Pacific states and even in New York, Wisconsin, Minnesota, North Dakota, South Dakota, and Nebraska other colored men, Indians, Chinese and Japanese outnumber the negro farmers. More than 60 per cent of the farmers of Mississippi are negroes, between 50 and 60 per cent in South Carolina, and between 40 and 50 per cent in Georgia, Alabama, and Louisiana. In the decade from 1900 to 1910 the value of farm property operated by negroes more than doubled.

As valuable as this report is, there are many questions about the negro left unanswered. In considering the welfare of the nation this race can not be left out of consideration. It is regrettable that he was brought here, but he was not consulted on this point and now that he is here to the number of about one-

tenth of the population, what is to be his future and what effect is he to have on the future of the white race? In making a forecast on these points the census compilation is of the greatest value, but we should like to know more. Absorption by the white race, indicated by increase in the number of mulattoes is a most interesting matter. We would like to know how the mulatto compares with the pure stock physically, mentally, and morally. Most biologists agree that for the betterment of the race two fundamental conditions are desirable: (1) Father and mother must not be too much alike, (2) Father and mother must not be too much unlike. For the first reason cousins of the first degree must not marry. As a consequence of the second law, we point to the mixed races of India, South and Central America and we conclude that the mongrel inherits the bad traits on both sides of the house. Does the mulatto comply with or violate this rule? Possibly in a later census more light on this point may be secured, but whatever the answer to this question may be it seems certain that the mulatto will multiply and one thousand years from now, if the world last so long, the black negro in this country will be as rare as a white elephant. In the meantime it seems quite evident that the mass of the race will remain in the South where there will be more or less segregation. In all probability the death rate will fall with improved sanitation and increased knowledge. In our opinion the most difficult medical problem in attempting to improve the race is that of the venereal diseases. Army statistics have shown for the first time the alarming prevalence of syphilis among the blacks. It is bad enough among whites, but among the blacks it is untreated and unless improvement be made, a few more generations may materially reduce the numbers. From the increase in mulattoes it is evident that syphilis among the blacks constitutes a serious menace to the whites as well.

*V. C. V.*